Kinetic Control of Mg$^{2+}$-dependent Melting of Duplex DNA Ends by *Escherichia coli* RecBC

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Received 19 December 2007; received in revised form 4 March 2008; accepted 13 March 2008

Available online 19 March 2008

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Abbreviations used: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; ITC, isothermal titration calorimetry.

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Keywords: fluorescence; motor protein; helicase; recombination; kinetics and thermodynamics

Introduction

Helicases are a class of motor enzymes that have critical roles in all aspects of DNA and RNA metabolism. These enzymes catalyze the separation of double-stranded (ds) DNA (or RNA) to form the single-stranded (ss) DNA intermediates required for DNA replication, recombination and repair via the coupling of energy from nucleoside triphosphate (NTP) binding and hydrolysis. To function processively, helicases must also translocate along the DNA filament. Such enzymes can also disrupt protein–DNA complexes, and this appears to provide an important biological function.

The *Escherichia coli* RecBCD helicase is responsible for the majority of recombinational repair at dsDNA breaks. RecBCD is a heterotrimeric enzyme consisting of the RecB (134 kDa), RecC (129 kDa) and RecD (67 kDa) subunits. Both the RecB and RecD subunits are superfamily 1 (SF1) DNA helicases, but unwind dsDNA with opposite polarities; RecB is a 3′ to 5′ helicase/translocase and RecD is a 5′ to 3′ helicase/translocase. RecBCD binds and
initiates DNA unwinding from a blunt or nearly blunt DNA end, the enzyme generates a 3'-ended ssDNA intermediate after encountering a recombination hotspot, called chi (\( \chi \) (S'–GCTGCTGTG-3'))\(^{15–17} \)

After chi recognition, RecBCD facilitates the loading of the RecA protein onto the unwound 3'-ssDNA.\(^{18} \)

The RecA-bound ssDNA filament then forms a joint molecule with a homologous region of DNA to initiate a recombination event. The RecBC enzyme, lacking the RecD subunit, can also function as a processive helicase and is capable of facilitating homologous recombination in vitro.\(^{19,20} \)

However, the nuclelease activity of RecBC is greatly attenuated,\(^{21–24} \)

even though the nuclease site is located within the 30 kDa C-terminal domain of the RecB subunit,\(^{25–27} \)

indicating that the nuclelease activity is stimulated by the RecD subunit.

Both RecBCD and RecBC enzymes initiate DNA unwinding from duplex ends, but they do not show a preference for blunt or single-strand over double-strand ends.\(^{28–33} \)

Studies of initiation complexes formed between RecBCD and a blunt-ended duplex DNA show that the RecB subunit can be crosslinked to the 3'-strand of the duplex end, while the RecC and RecD subunits can be crosslinked to the 5'-strand.\(^{34} \)

Furthermore, 5–6 bp at the duplex end within the RecBC–dsDNA initiation complex become accessible to KMnO\(_4\) attack in a Mg\(^{2+}\)-dependent but ATP-independent manner,\(^{35} \)

suggesting that RecBCD melts out or unwinds 4–5 bp upon binding to a blunt DNA end. A crystal structure of a RecBC–DNA complex, formed in the presence of Ca\(^{2+}\), but without ATP, shows a melting of ∼4 bp at the duplex DNA end.\(^{25} \)

Equilibrium binding of both RecBCD and RecBC to duplex DNA ends is enhanced if the DNA end possesses pre-formed 3' and/or 5' ssDNA flanking regions,\(^{36} \)

with RecBC showing optimal binding to a DNA end with both 3'-(dT)\(_6\) and 5'-(dT)\(_6\) tails, whereas RecBCD binds optimally to a DNA end with a 3'-(dT)\(_6\) tail, but a 5'-(dT)\(_{10}\) tail.\(^{37} \)

These results suggest that both enzymes are capable of disrupting ∼6 bp upon binding to a blunt duplex DNA end;\(^{37} \)

however, melting of a duplex DNA end by RecBCD has not been demonstrated. In the current study, we have compared the effects of Mg\(^{2+}\) on the equilibrium binding of RecBC to duplex DNA ends possessing variable lengths of pre-existing ssDNA tails as well as on the patterns of KMnO\(_4\) protection. These results demonstrate that RecBC is also able to melt out at least 4 bp upon binding to a duplex DNA end in a Mg\(^{2+}\)-dependent, but ATP-independent reaction. Our studies suggest also that the effect of Mg\(^{2+}\) is to relieve a kinetic block to DNA melting by RecBC, rather than to affect the equilibrium binding affinity of RecBC for the DNA.

Results

DNA substrate design

The experiments described here were performed using a series of 60 bp Cy3-labeled reference DNA (I–III) and unlabeled DNA molecules (IV–VI) shown schematically in Fig. 1a. The 60 bp duplex length ensures that one molecule of RecBC can bind independently to each duplex end without interference from protein binding to the other end.\(^{37} \)

The almost identical ends within each DNA duplex molecule simplify the data analysis, as RecBC binds to both ends with the same affinity within experimental error.\(^{37} \)

The equilibrium constant for RecBC binding to an end of a Cy3-labeled reference DNA molecule (I–III) is referred to here as \(K_{BC,R}\), while \(K_{BC}\) denotes the equilibrium constant for RecBC binding to an unlabeled duplex DNA end (IV–VI). The sequences of DNA strands used to form the duplex DNA molecules in Fig. 1a are given in Fig. 1c.

Effects of Mg\(^{2+}\) on RecBC binding to reference DNA I

Generally, an increase in the bulk solution [NaCl] will decrease the equilibrium binding constant for most protein–DNA interactions due to the fact that the Na\(^+\) counterion is displaced from the DNA when the protein binds.\(^{38–41} \)

If there is a mixture of Na\(^+\) and Mg\(^{2+}\) in the buffer, and if Mg\(^{2+}\) interacts only with the DNA and not the protein, then the Mg\(^{2+}\) should compete with both the Na\(^+\) and protein for binding to the DNA. Thus, if Mg\(^{2+}\) were only serving as a competitor for DNA binding, then the protein–DNA binding constant measured in the presence of Mg\(^{2+}\) should always be less than or equal to the binding constant measured in the absence of Mg\(^{2+}\) at the same [NaCl].\(^{42,43} \)

We examined the effects of Mg\(^{2+}\) on the [NaCl]-dependence of the equilibrium constant for RecBC binding to a duplex DNA end that we expect will be partially melted in the presence of Mg\(^{2+}\). For this purpose, we examined RecBC binding to reference DNA I, which has a Cy3 fluorophore on each 5'-end of the DNA and a 3'-Cy3-DNA end. On the basis of our previous studies,\(^{37} \)

RecBC binding to each end of this DNA is expected to melt an additional 2 bp in the presence of Mg\(^{2+}\). We first examined the dependence of the RecBC-DNA end binding constant \(K_{BC,R}\) on [NaCl] by performing salt-back titrations in the presence and in the absence of 10 mM MgCl\(_2\) as described\(^{44,45} \)

(see Materials and Methods) and the results are plotted in Fig. 2. At the starting [NaCl] of 0.10 M, RecBC binds with higher affinity to the DNA end in the presence of 10 mM MgCl\(_2\) (\(K_{BC,R} =4.8\) ± 0.3) × 10\(^7\) M\(^{-1}\) in 10 mM MgCl\(_2\) versus 2.3 ± 0.2) × 10\(^7\) M\(^{-1}\) in the absence of MgCl\(_2\)). Upon increasing the [NaCl] we observe a decrease in \(K_{BC,R}\) both in the presence and in the absence of 10 mM MgCl\(_2\). Plots of log \(K_{BC,R}\) versus log [NaCl] are linear within experimental error over the range from 0.1 to 0.85 M NaCl, with log-log slopes of −1.9 ± 0.4 in the presence of 10 mM MgCl\(_2\) and −4.5 ± 0.6 in the absence of MgCl\(_2\). Therefore, at any [NaCl], \(K_{BC,R}\) is always larger in the presence of 10 mM MgCl\(_2\) with the relative effect of MgCl\(_2\) increasing with increasing [NaCl] (Fig. 2).

This result is opposite to the expected result if Mg\(^{2+}\) were to bind only to the DNA and compete for bin-
of Na+ and RecBC since under those circumstances the values of $K_{BC,R}$ should converge, rather than diverge at high [NaCl].42,43 This result provides a clear indication that Mg2+ also binds directly to the RecBC protein and/or the RecBC–DNA complex, and facilitates RecBC binding to the DNA end. The simplest interpretation of the slopes of the plots in Fig. 2 indicates that approximately five ions (Na+ and/or Cl−) are released upon RecBC binding to an end of reference DNA I in the absence of MgCl2, while ~two ions (Na+ and/or Cl−) are released when RecBC binds to a DNA end in the presence of 10 mM MgCl2.

Independent experiments indicated that the fluorescence intensity of the Cy3-labeled DNA is unaffected by increases in [NaCl], thus enabling us to use salt-back titrations to obtain estimates of $K_{BC,R}$. As a further check, we measured $K_{BC,R}$ directly at 300 mM and 400 mM NaCl by determining a full binding isotherm (titrating RecBC into DNA I and monitoring the Cy3 fluorescence increase). The values of $K_{BC,R}$ determined directly (triangles in Fig. 2) and by the salt-back titration (circles in Fig. 2) agree within experimental uncertainty.

The pre-existing ss-(dT)n tail length influence the effects of Mg2+ on RecBC binding to DNA ends

The data in Fig. 2 show that $K_{BC,R}$ for RecBC binding to a DNA end with a 3′-(dT)n ssDNA tail is increased in the presence of 10 mM MgCl2. As mentioned above, we anticipate that 2 bp within the duplex region at the ss–dsDNA junction should be melted upon RecBC binding to such a DNA end. We next determined the relative equilibrium constants for RecBC binding to DNA ends possessing different lengths of pre-existing ss-(dT)n tails. For these studies, we measured equilibrium constants, $K_{BC}$, for
RecBC binding to the non-fluorescent DNA series IV (variable 3′-(dT)_n tail) and V (variable 5′-(dT)_n tail) in the presence and in the absence of 10 mM MgCl₂ using competition titration experiments. These experiments were performed by titrating a mixture of reference DNA I and one of the competitor DNA series molecules with RecBC as described. For each competitor DNA molecule, three experiments were performed in which the concentration of the non-fluorescent competitor DNA molecule was increased in each successive experiment. Data from all three experiments were analyzed globally to obtain K_{BC} using non-linear least-square (NLLS) methods as described, and the values of K_{BC} are given in Table 1. The competition binding isotherms obtained from a representative experiment performed using a DNA IV series molecule with n=8 nucleotides are shown in Fig. 3a. The relative fluorescence enhancement (ΔF_{obs}, defined in Eq. (2) in Materials and Methods) is plotted as a function of total [RecBC] together with simulated isotherms based on the best-fit values of K_{BC} (Table 1) as described.

Figure 3b shows plots of the ratio of K_{BC} measured in the absence of MgCl₂ to K_{BC} measured in the presence of 10 mM MgCl₂ as a function of the length of the pre-existing ss-(dT)_n tail. The dependences of K_{BC} on the length of the pre-existing ss-(dT)_n tail are qualitatively similar in the presence or in the absence of 10 mM MgCl₂ (Table 1). In both cases, a maximum in K_{BC} is observed for a DNA end with a pre-existing 3′-(dT)_n tail with n≥6 nucleotides. Importantly, the values of K_{BC} for the DNA IV and V series measured in the absence of MgCl₂ are lower than K_{BC} measured in 10 mM MgCl₂ when n<6 nucleotides (Table 1); however, for n≥6 nucleotides, K_{BC} is the same in the absence or in the presence of 10 mM MgCl₂. As shown in Fig. 3b, the difference between K_{BC} measured in the absence and in the presence of MgCl₂ decreases as the length of the pre-existing ss-(dT)_n tail increases from zero to 6 nt, with the values of K_{BC} becoming independent of the presence of Mg^{2+} for n≥6 nucleotides. The largest difference is observed for a blunt DNA end such that K_{BC} is about three times higher in the presence of 10 mM MgCl₂. We also observed no effect of 10 mM MgCl₂ on the values of K_{BC} for a DNA VI molecule possessing pre-existing twin ss-(dT)_n tails on both ends (open triangle in Fig. 3b). Hence, Mg^{2+} affects only K_{BC} for RecBC binding to a DNA end with a 3′- or 5′- (dT)_n tail if n<6 nucleotides. This suggests that the effect of Mg^{2+} is observed only when RecBC can...
tentially melt out some base pairs within a duplex DNA end.

**Effects of Mg\(^{2+}\) on melting of a DNA end by RecBC as examined by chemical protection of DNA**

Although the results of the binding studies described above suggest that RecBC melts some of the base pairs within the duplex DNA end region in the presence of Mg\(^{2+}\), we performed additional independent experiments to test this hypothesis. We performed KMnO\(_4\) footprinting experiments on the RecBC–blunt-ended-DNA complex to examine if base pairs are melted out in a Mg\(^{2+}\)-dependent manner. KMnO\(_4\) preferentially oxidizes the C5–C6 double bond within unstacked thymine bases within DNA \(^{46}\) and thus should detect melting of a duplex region containing thymidine base pairs. In fact, this approach was used by Farah & Smith \(^{35}\) to demonstrate that RecBCD melts out 4–5 bp upon binding to a blunt duplex end. We used a blunt-ended DNA (the same DNA used in the fluorescence titration experiments discussed above) radiolabeled with \(^{32}\)P at the 5’-end of the top strand (Fig. 4). In addition to performing the experiments in buffer M in the presence or in the absence of 10 mM MgCl\(_2\), we also performed experiments in both 30 mM and 100 mM NaCl to determine if this range of monovalent salt concentration influences base-pair melting. As shown in Fig. 4, the thymine base at position 4 (T4) is significantly more susceptible to KMnO\(_4\) attack in the presence of 10 mM MgCl\(_2\) and RecBC, regardless of the [NaCl]. This is the only thymine base within the 6 bp region from the end of the 5’-T\(_8\)-P-labeled strand. None of the other thymines at other positions (T8 and beyond in Fig. 4) exhibits any enhancement in susceptibility to KMnO\(_4\) attack in the presence of RecBC and 10 mM MgCl\(_2\). This indicates that at least 4 bp at the end of a blunt-ended DNA are melted upon binding of RecBC in a Mg\(^{2+}\)-dependent manner.

**Dependence of DNA melting by RecBC on the concentration of Mg\(^{2+}\)**

The effects of Mg\(^{2+}\) on the equilibrium constant for RecBC binding to a DNA end, \(K_{BC,R}\) and \(K_{BC}\), indicate that the binding of Mg\(^{2+}\) to RecBC increases its affinity for DNA ends containing pre-existing ss-(dT)\(_n\) tails only if the tails are shorter than 6 nt. The KMnO\(_4\) chemical protection experiments indicate that base-pair melting by RecBC is also dependent on the presence of Mg\(^{2+}\). Together, these results indicate that the binding of Mg\(^{2+}\) to RecBC and/or the RecBC–DNA complex is linked to base-pair melting by RecBC as has been demonstrated to be the case for RecBCD. \(^{35}\) To further study these two processes and to estimate the equilibrium constant for Mg\(^{2+}\) binding to the RecBC–DNA complex, we examined the fluorescence intensity of Cy3-labeled reference DNA molecules (DNA I through III) when pre-bound with RecBC as a function of [MgCl\(_2\)]. As shown in...
The position of the 32P label on the DNA. The reference are indicated in the figure. The asterisk in the inset shows ssDNA top strand alone. The contents of lanes 2 through 9 are indicated in the Methods. Lane 1 is a control with the (5\textprime;-(dT)6) tail and DNA band is indicated by ref and the uncut sample DNA band is denoted by uncut.

![Diagram](image.png)

**Fig. 4.** Effects of Mg2+ on the chemical protection patterns of a blunt-ended DNA bound by RecBC. KMnO4 footprinting experiments of the RecBC-blunt-ended-DNA complex were performed in buffer M plus the indicated [NaCl] and [MgCl2] at 25 °C as described in Materials and Methods. Lane 1 is a control with the (5\textprime;-(dT)6) and DNA with RecBC in the absence of MgCl2 (buffer M plus 100 mM NaCl at 25 °C) and then titrating with MgCl2 while monitoring the Cy3 fluorescence signal.

The results of these experiments are presented in Fig. 5a, where the corrected Cy3 fluorescence (F_{corr}) as defined in Eq. (1) in Material and Methods) is plotted as a function of the total [MgCl2]. The fluorescence intensities of Cy3 within the RecBC–DNA I and RecBC–DNA II complexes were enhanced upon titrating with MgCl2. In contrast, the Cy3 fluorescence signal of the RecBC-bound reference DNA III, which has twin ss-(dT)6 tails on both ends, does not change upon addition of MgCl2. The largest enhancement in Cy3 fluorescence (∼58%) is observed for the RecBC-bound DNA I, which has a 3\textprime;-(dT)4 tail, while the RecBC-bound DNA II, which has twin ss-(dT)2 tails, exhibits a smaller Cy3 fluorescence enhancement (∼21%). Since the Cy3 fluorescence of reference DNA alone is independent of [MgCl2] (data not shown), the observed enhancement of the Cy3 fluorescence signals of the RecBC–DNA I and RecBC–DNA II complexes upon titration with MgCl2 is due to the effects of Mg2+ on the RecBC–reference DNA complexes. The final fluorescence levels for all RecBC–reference DNA complexes are the same within experimental error, indicating that the final environments of the Cy3 fluorophores in all RecBC–DNA complexes are the same. The fact that only the reference DNA with ss-(dT)6 tails shorter than 6 nt (DNA I and DNA II) exhibit fluorescence enhancement suggests that the increase in Cy3 fluorescence is due to DNA melting. No enhancement of the Cy3 signal was observed for reference DNA III, which has twin ss-(dT)2 tails, consistent with the expectation that no additional base-pair melting should occur upon RecBC binding.

We also performed a MgCl2 titration of DNA I and DNA II in the presence of 400 mM NaCl, since this higher [NaCl] should further reduce Mg2+ binding to the DNA. As shown in Fig. 5a, the results obtained in 400 mM NaCl are identical with the those obtained in 100 mM NaCl, thus the binding of Mg2+ to the DNA appears to be weak enough at these high [NaCl] that it does not compete with the binding of Mg2+ to the RecBC–DNA complex. To examine this further, we calculated the expected extent of Mg2+ binding to a DNA duplex under these concentrations of salt using the values of the Mg2+ binding constant determined as a function of [NaCl] from the study of non-specific interactions between lac repressor and calf thymus DNA as well as pentaylsine and T7 DNA. On the basis of these results one can calculate the probability (P_{Na}) that a nucleotide within the duplex DNA has only Na+ and no Mg2+ associated with it when the duplex DNA is placed in a buffer containing both Na+ and Mg2+ (see Appendix). These calculations indicate that at 100 mM NaCl, P_{Na} ≈ 0.62 at the end of the titration when 10 mM MgCl2 is present. At 400 mM NaCl, P_{Na} ≈ 0.95 at the end of the titration when 10 mM MgCl2 is present. We also note that the equilibrium constants for Mg2+ binding to duplex DNA used in the above calculations were determined for Mg2+ binding to long duplex DNA. Since the DNA used in our experiments is only 60 bp long, there will be less Mg2+ binding to the shorter DNA than estimated from the calculations. Therefore, we
conclude that there should be relatively little Mg\(^{2+}\) bound to the DNA even at 10 mM Mg\(^{2+}\), especially at 400 mM NaCl. Although these results suggest that the binding of Mg\(^{2+}\) to the RecBC–DNA complex is relatively insensitive to the [NaCl], this may be complicated by any compensating effects due to the [NaCl]-dependence of Mg\(^{2+}\) binding to the DNA.

As shown in Fig. 5a, the midpoint of the Mg\(^{2+}\) titrations for DNA I performed in either 100 mM or 400 mM NaCl is \(\sim 1.6(\pm 0.4)\) mM MgCl\(_2\) while titrations for DNA II exhibit a midpoint of \(1.8(\pm 0.4)\) mM MgCl\(_2\). This indicates that the melting of base pairs in both DNA I and II has the same dependence on the concentration of MgCl\(_2\) despite the expectation that a different number of base pairs are melted in DNA I versus II (2 bp versus 4 bp, respectively).

To obtain estimates of the apparent equilibrium constant for Mg\(^{2+}\) binding to the RecBC and RecBC–DNA complex, we analyzed the MgCl\(_2\) titrations quantitatively using a simple model that assumes only one Mg\(^{2+}\)-binding site per RecBC (see Scheme 1, and Materials and Methods). This model ignores any binding of Mg\(^{2+}\) to duplex DNA; however, as discussed above, this is expected to be small at the high [NaCl] (100 mM and 400 mM) used in our experiments. NLLS analysis of the data using Eq. (17) (see Materials and Methods) yields a value of \(K_{Mg}^{BD} = 5(\pm 2) \times 10^2\) M\(^{-1}\) for the equilibrium constant for Mg\(^{2+}\) binding to either a RecBC–DNA I or a RecBC–DNA II complex, and a value of \(K_{Mg}^{B} = 8(\pm 3)\) M\(^{-1}\) for the equilibrium constant for Mg\(^{2+}\) binding to RecBC, assuming only one Mg\(^{2+}\) binding site per RecBC.

Somewhat surprisingly, Ca\(^{2+}\) has identical effects on the Cy3 fluorescence signals of RecBC–DNA complexes as Mg\(^{2+}\). As shown in Fig. 5b, the traces obtained from titrations of the RecBC–DNA I complex with CaCl\(_2\) and MgCl\(_2\) are identical within experimental uncertainty. Titration of the RecBC–DNA III complex with CaCl\(_2\) also exhibits no change in Cy3 fluorescence intensity (Fig. 5b). We also observed that RecBC binds to reference DNA I with the same affinity \((K_{BC,R} = 4.8(\pm 0.5) \times 10^7\) M\(^{-1}\)) in either 10 mM MgCl\(_2\) or 10 mM CaCl\(_2\) (data not shown). These data indicate that the observed equilibrium constant for the RecBC–DNA complex binding to one calcium ion is identical with that for Mg\(^{2+}\). Even though a crystal structure of RecBCD bound to a duplex DNA in the presence of Ca\(^{2+}\) shows that RecBCD can melt out 4 bp at the end of the duplex, our quantitative studies are somewhat surprising, since Mg\(^{2+}\) and Ca\(^{2+}\) binding sites on proteins are expected to be different.

Since a calcium ion is observed bound at the RecB nuclease domain active site in the crystal structure of

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**Fig. 5.** Effects of Mg\(^{2+}\) on the Cy3 fluorescence signal of a RecBC-reference DNA complex. (a) 10 nM DNA I (●), DNA II (○) or DNA III (◊) was pre-bound with 2.4 μM, 2.2 μM or 1.3 μM RecBC, respectively, and titrated with MgCl\(_2\) in buffer M plus 100 mM NaCl at 25 °C and the corrected Cy3 fluorescence \(F_{corr}\) is plotted as a function of total [MgCl\(_2\)]. The same experiments were performed in buffer M plus 400 mM NaCl for DNA I (□) and DNA II (○). Continuous lines are simulations using Eqs. (13)–(15) and the best fit values of \(K_{BD,1}^{Mg} = 5(\pm 2) \times 10^2\) M\(^{-1}\) and \(8(\pm 3)\) M\(^{-1}\), respectively. (b) Comparisons of 10 nM DNA I pre-bound with 2.4 μM RecBC titrated with MgCl\(_2\) (●) or CaCl\(_2\) (×) in buffer M plus 100 mM NaCl at 25 °C. \(F_{corr}\) is plotted as a function of total [MgCl\(_2\)] or [CaCl\(_2\)]. 10 nM DNA III pre-bound with 1.3 μM RecBC was also titrated with CaCl\(_2\) (○) in buffer M plus 100 mM NaCl at 25 °C. \(F_{corr}\) is plotted as a function of total [MgCl\(_2\)] or [CaCl\(_2\)]. (c) Comparisons of 10 nM DNA I pre-bound with either 2.4 μM RecBC (●) or 2.4 μM RecB\(^{Δnuc}\) (▽) and titrated with MgCl\(_2\) in buffer M plus 100 mM NaCl at 25 °C. \(F_{corr}\) from each experiment was normalized arbitrarily to 1 and plotted as a function of [MgCl\(_2\)].
RecBC–DNA complex, we next tested if this site is responsible for the observed binding of Mg$^{2+}$ by examining RecB$^{ΔnucC}$, which was reconstituted from RecC and a RecB nuclease domain deletion mutant (RecB$^{Δnuc}$). Fig. 5c shows that the [MgCl$_2$]-dependence of the normalized enhancement of the Cy3 fluorescence signal of a RecB$^{Δnuc}$-DNA I complex is the same as observed for the normalized Cy3 fluorescence signal for a RecBC–DNA I complex. Therefore, the nuclease domain of RecB is not responsible for the observed effect of Mg$^{2+}$ on DNA melting.

**Effects of Mg$^{2+}$ on the observed enthalpy and heat capacity changes for RecBC binding to DNA ends**

As shown above, for RecBC binding to a DNA end possessing a pre-existing twin-ss-(dT)$_6$ tail (DNA VI), there is no effect of Mg$^{2+}$ on $K_{BC}$ and thus none on the standard state binding free energy change, $ΔG^{mic}$ = $−R$T$ln K_{BC}$ (Fig. 3b and Table 1). While this suggests that there is no effect of Mg$^{2+}$ on the energetics of the RecBC–DNA VI (n = 6) interaction, it is possible that there is an enthalpy/entropy compensation under the conditions used resulting in similar values of $K_{BC}$. We therefore performed isothermal titration calorimetry (ITC) experiments to measure $ΔH_{obs}$ for RecBC binding to the ends of a DNA VI molecule with n = 6 in the presence and in the absence of 10 mM MgCl$_2$ over a temperature range of 5–25 °C. We also compared the $ΔH_{obs}$ for RecBC binding to a DNA VI molecule with n = 6, for which no base-pair melting is expected, to that for RecBC binding to a blunt-ended DNA molecule (DNA VI with n = 0) for which base-pair melting is expected to occur only in the presence of Mg$^{2+}$.

The results of two representative ITC experiments are shown in Figs. 6a and b, where the heat of each injection normalized to the amount of DNA injected ($ΔQ_{norm}$ as defined in Eq. (20) in Materials and Methods) is plotted as a function of the ratio of total [DNA]/[total [RecBC] (buffer M, 100 mM NaCl). The data were analyzed (see Eqs. (18)–(20) in Materials and Methods) and the values of the observed enthalpy change ($ΔH_{obs}$) for RecBC binding to one DNA end are presented in Table 2 and Fig. 6c. In the temperature range 5–25 °C, $ΔH_{obs}$ for RecBC binding to a DNA end containing twin-ss-(dT)$_6$ tails (DNA VI with n = 6) is the same within experimental error, in the absence or in the presence of 10 mM MgCl$_2$. This indicates that the complete thermodynamic profile ($ΔG_{obs}$, $ΔH_{obs}$, $ΔS_{obs}$ and $ΔC_P_{obs}$) for RecBC binding to the pre-melted ends of DNA VI (with n = 6) is identical in the absence or in the presence of MgCl$_2$. Hence, Mg$^{2+}$ has no effect on the energetics of RecBC binding to a fully pre-melted DNA end over the temperature range 5–25 °C. RecBC binding to the DNA with twin-ss-(dT)$_6$ tails (DNA VI with n = 6) exhibits the same $ΔC_{P,obs}$ in the presence or in the absence of 10 mM MgCl$_2$: $−1.6(±0.3)$ kcal mol$^{-1}$ K$^{-1}$, respectively.

On the other hand, we observe a definite effect of Mg$^{2+}$ on the values of $ΔH_{obs}$ and its heat capacity change ($ΔC_{P,obs}$ = $dΔH_{obs}/dT$)$_p$ for RecBC binding to a blunt DNA end (DNA VI with n = 0) (Table 2 and Fig. 6c) measured over the temperature range 15–25 °C. Interestingly, $ΔC_{P,obs}$ for RecBC binding to a blunt-ended DNA in the presence of 10 mM MgCl$_2$ is very similar, $−1.2(±0.2)$ kcal mol$^{-1}$ K$^{-1}$, to $ΔC_{P,obs}$ for RecBC binding to the twin-ss-(dT)$_6$ tailed DNA, although the values of $ΔH_{obs}$ are much smaller in magnitude for RecBC binding to the blunt DNA end. In contrast, $ΔC_{P,obs}$ for RecBC binding to a blunt-ended DNA in the absence of MgCl$_2$ is $−0.5(±0.3)$ kcal mol$^{-1}$ K$^{-1}$, about 2.5 times smaller. Hence, a larger negative value of $ΔC_{P,obs}$ is observed under conditions where the final state of the DNA has six unpaired bases when bound to RecBC.

We also used the competition fluorescence titration experiments to examine the effects of 10 mM MgCl$_2$ on $K_{BC}$ for RecBC binding to a blunt DNA end (DNA VI with n = 0) at 5 °C, 15 °C and 25 °C and compared these values with the predicted temperature-dependence of $K_{BC}$ based on the $ΔC_{P,obs}$ and $ΔH_{obs}$ values obtained from the ITC studies (Table 2). The results are presented in Table 3 and plotted in Fig. 6d. The lines in Fig. 6d are simulations using Eq. (22) and the $ΔH_{obs}$ and $ΔC_{P,obs}$ values from Table 2. As shown in Fig. 6d, there is excellent agreement between the measured values of $ln K_{BC}$ both in the presence and in the absence of MgCl$_2$, and those calculated using the parameters obtained from the ITC studies.

**Enthalpic cost of base-pair melting**

We next performed a series of experiments to obtain an estimate of $ΔH_{obs}$ for melting a base pair within the RecBC–DNA end complex. For this purpose, we measured $ΔH_{obs}$ for RecBC binding to the ends of the series of DNA VI molecules containing twin ss-(dT)$_n$ tails, with n = 0, 2, 4, 6, 8, 10 or 20, in the presence of 10 mM MgCl$_2$. We reasoned that, in the presence of MgCl$_2$, 6 bp should be melted upon RecBC binding to a blunt DNA end (DNA VI with n = 0), while no base pair should be melted upon RecBC binding to a DNA end with twin ss-(dT)$_n$ tails, where n ≥ 6. Thus, the difference between $ΔH_{obs}$ for RecBC binding to a blunt DNA end and $ΔH_{obs}$ for RecBC binding to a DNA end with twin ss-(dT)$_n$ tails should provide a measure of the enthalpic cost for melting 6 bp. Of course, this assumes that the energetic state of the final RecBC–DNA complex (with 6 bp melted) is unaffected by DNA base composition.

The results of these experiments are presented in Table 4 and Fig. 7. $ΔH_{obs}$ for RecBC binding to one

\[ 2B + D \xrightleftharpoons{K_{BC}} B + BD \xrightleftharpoons{K_{D}} B + D \]

\[ 2\text{Mg}^{2+} \]
end of DNA VI is always negative and decreases linearly from −17(±4) kcal mol⁻¹ to −64(±3) kcal mol⁻¹ as the lengths of the pre-formed twin ss-(dT)ₙ tails increase from zero to 6 nt; however, ΔH(obs) remains unchanged at −64(±3) kcal mol⁻¹ for n ≥ 6 nucleotides. A linear fit to the length dependence of ΔH(obs) for n = 0–6 yields a value of −8(±1) kcal mol⁻¹. Therefore, the average enthalpic cost of melting one DNA base pair by RecBC is 8(±1) kcal mol⁻¹ bp⁻¹.

**Discussion**

Previous studies of RecBCD binding to a blunt DNA duplex end have shown that the last 4–5 bp at the end of the duplex become accessible to KMnO₄ attack in a Mg²⁺-dependent but ATP-independent manner.³⁵ The last 4 bp of a blunt-ended duplex DNA are observed to be unpaired in a crystal structure of a RecBCD–DNA complex formed in the presence of Ca²⁺.²⁷ Equilibrium binding studies performed in the absence of ATP also show that RecBCD binds tightest to a DNA end containing pre-existing 3'-ss-(dT)ₙ and 5'-ss-(dT)ₙ tails.³⁷ These observations indicate that RecBCD is capable of melting out the last 4–6 bp upon binding to a blunt duplex DNA end in a divalent cation-dependent, but ATP-independent reaction. Here, we have shown that RecBC is able to carry out a similar base-pair melting reaction upon binding a blunt-ended DNA.

**Base-pair melting by RecBC is Mg²⁺-dependent**

Divalent cations such as Mg²⁺ can always compete with monovalent cations and protein for the binding of DNA, and thus the presence of Mg²⁺ will generally decrease the equilibrium constant for protein binding to DNA and the magnitude of its dependence on [NaCl].⁴⁰,⁴¹ Although for RecBC binding to DNA I we observe that the dependence of the equilibrium constant (K_BC,R) on [NaCl] is affected by the presence of 10 mM MgCl₂, the effects are very different from those expected if Mg²⁺ acted only as a competitor. The fact that K_BC,R measured in the presence of

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**Fig. 6.** Effects of Mg²⁺ on the temperature dependence of the observed enthalpic change (ΔH(obs)) for RecBC binding to one end of the DNA VI series molecules with n = 0 or 6. Experiments were performed in buffer M plus 100 mM NaCl and the indicated [MgCl₂] at the indicated temperature. (a and b) Representative ITC experiments to determine the enthalpic change for RecBC binding the ends of DNA VI molecules with n = 0 or 6 in the presence of 10 mM MgCl₂ at 25 °C. The heat of each injection normalized to the amount of DNA injected (ΔQ/Δqₐₚₖ as defined in Eq. (20)) is plotted as a function of total [DNA]/total [RecBC]. (a) 710 nM RecBC was titrated with 15.2 μM DNA VI with n = 0; (b) 885 nM RecBC was titrated with 9 μM DNA VI with n = 6. Continuous lines are simulations using Eqs. (18)–(20) and the best fit values of ΔH(obs), (Table 2) and K_BC,R = (1.6 ± 0.3) × 10⁷ M⁻¹ in a, while K_BC,R ≥ 10⁹ M⁻¹ in (b). (c) Effects of Mg²⁺ on values of ΔC_p,obs for RecBC binding to a DNA end. ΔH(obs) for RecBC binding to an end of DNA VI with n = 6 in the presence of 10 mM MgCl₂ (●) or in the absence of MgCl₂ (○) and ΔH(obs) for RecBC binding to a blunt DNA end in the presence of 10 mM MgCl₂ (●) or in the absence of MgCl₂ (○) are plotted as a function of temperature (°C). Straight lines represent results obtained from linear least-squares analysis of each set of data and the value of ΔC_p,obs obtained from each set of data is presented in Table 2. (d) Effects of Mg²⁺ on the temperature dependence of equilibrium constant (K_BC,R) for RecBC binding to a blunt DNA end measured by competition fluorescence titration experiments. Experiments were performed in buffer M, 100 mM NaCl with or without 10 mM MgCl₂ at the indicated temperature. Values of ln K_BC,R measured with 10 mM MgCl₂ (●) or without MgCl₂ (○) are plotted as a function of temperature (°C). The continuous and broken lines are simulations using Eq. (22), and the ΔH(obs) and ΔC_p,obs values obtained from ITC experiments in the presence or in the absence of 10 mM MgCl₂ respectively (Table 2).
Table 2. Temperature-dependence of the observed enthalpic change (ΔHobs) for RecBC binding to one end of the DNA VI series molecules with n = 0 or 6

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>DNA with twin (dT)_n tails</th>
<th>Blunt-ended DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔHobs (kcal mol⁻¹)</td>
<td>ΔHobs (kcal mol⁻¹)</td>
</tr>
<tr>
<td></td>
<td>10 mM MgCl₂</td>
<td>no MgCl₂</td>
</tr>
<tr>
<td>5</td>
<td>-32±3</td>
<td>-31±3</td>
</tr>
<tr>
<td>10</td>
<td>-38±3</td>
<td>-35±3</td>
</tr>
<tr>
<td>15</td>
<td>-48±3</td>
<td>-47±3</td>
</tr>
<tr>
<td>17</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>20</td>
<td>-56±3</td>
<td>-53±2</td>
</tr>
<tr>
<td>25</td>
<td>-64±3</td>
<td>-61±2</td>
</tr>
</tbody>
</table>

ss-(dT)_n tail length (n) ΔCp,obs (kcal mol⁻¹ K⁻¹) 10 mM MgCl₂ ΔCp,obs (kcal mol⁻¹ K⁻¹) no MgCl₂

0               -1.2±0.2     -0.5±0.3
6               -1.6±0.3     -1.6±0.4

Observed heat capacity change (ΔCp,obs) values are obtained from linear least-squares analyses of the observed enthalpic change data. (buffer M plus 100 mM NaCl and the indicated [MgCl₂]). ND, not determined.

Table 3. Temperature dependence of the equilibrium constants (KBC) for RecBC binding to a blunt-ended DNA

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>KBC (10^7 M⁻¹) 10 mM MgCl₂</th>
<th>KBC (10^7 M⁻¹) no MgCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2.0±0.7</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>15</td>
<td>3.7±0.7</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>25</td>
<td>1.6±0.3</td>
<td>0.56±0.05</td>
</tr>
</tbody>
</table>

Buffer M plus 100 mM NaCl and the indicated [MgCl₂].

Mg²⁺ is always higher than KBC,R measured in the absence of Mg²⁺ at all [NaCl] examined indicates that Mg²⁺ facilitates RecBC binding to DNA. This Mg²⁺-dependent increase in KBC,R is consistent with the hypothesis that RecBC requires Mg²⁺ in order to melt a region of the duplex DNA end upon binding.

The effects of varying the lengths of the pre-existing tails on KBC for the DNA IV and V series in the absence of MgCl₂ are qualitatively similar to the effects observed in the presence of 10 mM MgCl₂. Therefore, these data by themselves cannot be used to conclude whether RecBC melts out 6 bp in a Mg²⁺-dependent manner. However, the quantitative differences between KBC measured in the absence and in the presence of MgCl₂ are consistent with the hypothesis that 6 bp at the blunt DNA end are melted by RecBC, failed to show an increase in the Cy3 fluorescence, whereas both RecBC-bound DNA I and II (with ss-DNA tails shorter than six nucleotides) exhibit an enhancement in Cy3 fluorescence upon titration of MgCl₂ consistent with base-pair melting being associated with the fluorescence enhancement upon addition of MgCl₂. The same final fluorescence level was exhibited by all RecBC-DNA complexes at the end of titration, indicating that all the RecBC–DNA complexes are in the same final state (i.e., 6 bp at the end are unpaired) at the end of titration.

Mg²⁺ overcomes a kinetic barrier to facilitate base-pair melting by RecBC

For RecBC-induced melting of duplex DNA to occur, the favorable free energy change accompanying RecBC binding to DNA (ΔG°bind) must be sufficient to overcome the unfavorable free energy change.
RecBC binding to one end of the DNA is plotted as a function of pre-existing ss-(dT) for this process is if Mg$^{2+}$ increases the affinity of Mg$^{2+}$ talic change (\(\Delta H_{\text{obs}}\)) for RecBC binding to one end of the DNA VI series containing twin ss-(dT) tails with \(n\) varying from zero to 20 nt were measured in buffer M plus 10 mM MgCl$_2$ and 100 mM NaCl at 25 °C. \(\Delta H_{\text{obs}}\) for RecBC binding to one end of the DNA VI series (●) are plotted as a function of pre-existing ss-(dT)$_n$ tail length (\(n\)).

Associated with base-pair melting (\(\Delta G_{\text{melt}}^0\)). One potential explanation for the requirement of Mg$^{2+}$ for this process is if Mg$^{2+}$ increases the affinity of RecBC for the fully melted DNA product so that \(\Delta G_{\text{bind}}^0 + \Delta G_{\text{melt}}^0 < 0\), whereas in the absence of Mg$^{2+}$ \(\Delta G_{\text{bind}}^0 + \Delta G_{\text{melt}}^0 > 0\). However, the fact that \(\Delta G_{\text{obs}}^0\) (\(-RT\ln K_{\text{BC}}\)) for RecBC binding to a DNA end possessing pre-existing ss-(dT)$_n$ tails with \(n \geq 6\) nucleotides is the same with or without 10 mM MgCl$_2$ indicates that there is already sufficient binding free energy available from the RecBC-DNA interaction even in the absence of Mg$^{2+}$, to achieve a melted structure. Yet, base-pair melting by RecBC does not occur in the absence of Mg$^{2+}$. Therefore, our results suggest that base-pair melting by RecBC binding to DNA is thermodynamically favored in the presence and in the absence of Mg$^{2+}$, but is kinetically blocked in the absence of Mg$^{2+}$.

Interestingly, the Cy3 fluorescence data suggest that Ca$^{2+}$ can also facilitate base-pair melting by RecBC. This observation is consistent with the fact that 4 bp are unpaired in the RecBCD-blunt-ended DNA crystal structure formed in the presence of Ca$^{2+}$. The one surprising observation is that the effect of Ca$^{2+}$ is indistinguishable from that of Mg$^{2+}$. Even if Ca$^{2+}$ and Mg$^{2+}$ bind to the same site to facilitate base-pair melting, it is not expected that they would have the same affinity for the same site on RecBC or the RecBC-DNA complex, given the distinctly different ion sizes and requirements for Ca$^{2+}$ and Mg$^{2+}$ binding sites within proteins. In fact, Ca$^{2+}$ is observed bound in the expected Mg$^{2+}$ binding site within the RecB nuclease domain, and does inhibit the nuclease activity of RecBC. It is possible that there are multiple sites for Mg$^{2+}$ and Ca$^{2+}$, and that there is a fortuitous compensation of effects such that the apparent affinity of Mg$^{2+}$ and Ca$^{2+}$ appear to be the same. Since both Ca$^{2+}$ and Mg$^{2+}$ appear to function to relieve a kinetic block associated with DNA melting by RecBC and RecBCD, this could mask any difference in affinity of these two ions for the RecBCD-DNA complex.

A calcium ion is observed bound at the Mg$^{2+}$-binding site within the nuclease domain in the crystal structure of a RecBCD-DNA complex. Yet, our data indicate that deletion of the nuclease domain to form RecB$\text{Δnuc}$C has no influence on the ability of Mg$^{2+}$ to facilitate DNA melting by RecBC. Therefore, the site for Mg$^{2+}$ binding that facilitates base-pair melting by RecBC is not located on the nuclease domain of RecB. The ATP-binding site on RecB is a potential site for binding Mg$^{2+}$ but it is possible that the Mg$^{2+}$-binding site is present on the RecBC subunit. The structural fold of RecC is similar to that of RecB, and it has been suggested that RecC may have been a defunct RecB helicase.

Thermodynamics of RecBC–DNA complex formation and base-pair melting

We observe that \(\Delta C_{p,\text{obs}}\) of \(-1.2(\pm 0.2)\) kcal mol$^{-1}$ K$^{-1}$ for RecBC binding to a blunt DNA end in the presence of Mg$^{2+}$ is very similar to the \(\Delta C_{p,\text{obs}}\) \(-1.6(\pm 0.3)\) kcal mol$^{-1}$ K$^{-1}$ with 10 mM MgCl$_2$ and \(-1.6(\pm 0.4)\) kcal mol$^{-1}$ K$^{-1}$ with no MgCl$_2$ for RecBC binding to a DNA end possessing two pre-existing twin-(dT)$_n$ tails (DNA VI with \(n = 6\) nt). This similarity suggests that there is little heat capacity change associated with base-pair melting, at least as it occurs within the RecBC complex. This is consistent with the conclusion that unstacking of bases does not contribute significantly to the \(\Delta C_{p,\text{obs}}\) of duplex disruption. In contrast, \(\Delta C_{p,\text{obs}}\) for RecBC binding to blunt DNA end in the absence of Mg$^{2+}$ is less negative at \(-0.5(\pm 0.3)\) kcal mol$^{-1}$ K$^{-1}$. The potential origins of the heat capacity change associated with any protein–DNA complex or any macromolecular interaction are numerous, and a determination of the origins of \(\Delta C_{p,\text{obs}}\) for this system are beyond the scope of this work. Further studies are required to elucidate the linked equilibria responsible for the large and negative \(\Delta C_{p,\text{obs}}\) for RecBC binding to DNA ends, and the difference between \(\Delta C_{p,\text{obs}}\) for the formation of the “melted” versus “un-melted” complexes.

We estimated the average enthalpic cost of melting 6 bp at a blunt DNA end by RecBC in the presence of Mg$^{2+}$ from the measurements of \(\Delta H_{\text{obs}}\) for RecBC binding to a DNA end possessing pre-existing twin ss-(dT)$_n$ tails (DNA VI) with \(n\) varying from 0 to 6 nt. This estimate is based on two assumptions. The first is that the end state of a RecBC–DNA complex is the same for \(0 \leq n \leq 6\) in the presence of 10 mM MgCl$_2$ i.e. the last 6 bp are unpaired in 100% of the RecBC–DNA complexes. The second assumption is that \(\Delta H_{\text{obs}}\) is independent of base sequence and composition. With these assumptions, we estimate a value of \(47(\pm 7)\) kcal mol$^{-1}$ for the enthalpic cost to melt out the last 6 bp at the blunt DNA end used in our experiments, which consists of four G/C and two A/T base-pairs. This corresponds to a value of
8(±1) kcal mol\(^{-1}\) bp\(^{-1}\) for the average enthalpic cost of melting out one base pair at 25 °C. Since this estimate is based on the difference in \(\Delta H_{obs}\) for binding of a series of RecBC–DNA VI complexes, the contributions to \(\Delta H_{obs}\) from the RecBC–DNA interactions should cancel out if the final RecBC–DNA complexes are the same and independent of base composition. Previous estimates of \(\Delta H_{obs}\) for base-pair melting are 4.3–9 kcal mol\(^{-1}\) bp\(^{-1}\),\(^{50}\) 5.2–15 kcal mol\(^{-1}\) bp\(^{-1}\),\(^{51}\) and –7 kcal mol\(^{-1}\) bp\(^{-1}\) from the nearest-neighbor model.\(^{35}\) Our value of 8(±1) kcal mol\(^{-1}\) bp\(^{-1}\) falls within this range. Since determination of \(\Delta H_{obs}\) for base-pair melting from DNA melting experiments is generally difficult due to the uncertainties associated with obtaining accurate baselines at low and at high temperatures, our determination may represent a more accurate estimate of this average quantity.

### Implications for the helicase mechanism of RecBC and RecBCD

We have shown that when RecBC binds the end of a blunt-ended DNA, it melts out 6 bp at the DNA end in a Mg\(^{2+}\)-dependent but ATP-independent manner. Our results suggest that Mg\(^{2+}\) functions by overcoming a kinetic barrier to the RecBC-mediated DNA melting process. The binding of RecBCD to a blunt DNA end also results in the unpairing of the last 4–5 bp at the end of the duplex DNA.\(^{35}\) The number of base pairs melted out by RecBCD upon binding to a blunt DNA end is very similar to the “kinetic step size” of 3.9(±0.6) bp s\(^{-1}\) estimated for RecBCD unwinding of DNA from pre-steady-state kinetic studies of DNA.\(^{31–33}\) Recall that a kinetic unwinding step size of 4 bp indicates that some rate-limiting step in the unwinding process is repeated every 4 bp, on average, during the unwinding process. The similarity between these two values suggests that DNA unwinding by RecBCD may occur in a two-step process in which 4–6 bp of DNA are melted upon binding of RecBCD to the duplex region independent of ATP, followed by more rapid ATP-dependent translocation of RecBCD to the newly formed ss/dsDNA junction.\(^{31–33}\) Since RecBC also melts out base pairs upon binding to a duplex DNA end, it is possible that RecBC unwinds DNA by the same mechanism, such that the RecBC binding alone is sufficient to actively open the next 4 bp.

### Materials and Methods

#### Buffers

Buffers were made from reagent-grade chemicals using doubly distilled water that was further detoxified with a Milli-Q purification system (Millipore Corp., Bedford, MA). Buffer C is 20 mM potassium phosphate (pH 6.8), 0.1 mM 2-mercaptoethanol, 0.1 mM EDTA, 10% (v/v) glycerol. Buffer M is 20 mM Mops–KOH (pH 7.0), 1 mM 2-mercaptoethanol, 5% (v/v) glycerol. The concentration of MgCl\(_2\) stocks was determined by measuring the refractive index of a stock solution in water using a refractometer (Mark II Leica Inc., Buffalo, NY) and a standard table relating refractive index to [MgCl\(_2\)].\(^{52}\)

### Proteins

*E. coli* RecB and RecC proteins were purified and reconstituted to form RecBC as described.\(^{63}\) RecB\(^{nuc}\) was purified and reconstituted with RecC to form RecB\(^{nuc}\)C as described.\(^{64}\) RecBC and RecB\(^{nuc}\)C concentrations were determined spectrophotometrically in buffer C using extinction coefficients of \(\varepsilon_{280}=3.9 \times 10^5\) M\(^{-1}\) cm\(^{-1}\) and \(\varepsilon_{280}=3.4 \times 10^5\) M\(^{-1}\) cm\(^{-1}\), respectively. All protein concentrations reported refer to the RecBC or RecB\(^{nuc}\)C heterodimer. Bovine serum albumin (BSA) was from Roche (Indianapolis, IN) and its concentration was determined using an extinction coefficient of \(\varepsilon_{280}=4.3 \times 10^4\) M\(^{-1}\) cm\(^{-1}\) in buffer C.\(^{37}\) All proteins were dialyzed into the appropriate reaction buffer before use. Dialyzed RecBC or RecB\(^{nuc}\)C were stored at 4 °C for up to five days, since a loss of activity (~15%) was observed after five days at 4 °C.

### Oligodeoxynucleotides

Oligodeoxynucleotides were synthesized using an ABI synthesizer (model 391, Applied Biosystems, Foster City, CA) using reagents and phosphoramidites from Glen Research (Sterling, VA). A first purification step of each single-stranded oligodeoxynucleotide was performed using polyacrylamide gel electrophoresis under denaturing conditions followed by recovery of the DNA from the gel by electroelution.\(^{62}\) The resulting oligodeoxynucleotides were then further purified chromatographically by reverse phase HPLC using an X Terra MS C18 column (Waters, Milford, MA). The concentration of each DNA strand was determined by completely digesting the strand with phosphodiesterase I (Worthington, Lakewood, NJ) in 100 mM Tris–HCl (pH 9.2), 3 mM MgCl\(_2\), at 25 °C and measuring the absorbance of the resulting mixture of mononucleotides at 260 nm as described.\(^{35}\) The extinction coefficients at 260 nm used in this analysis are: 15,340 M\(^{-1}\) cm\(^{-1}\) for AMP, 7600 M\(^{-1}\) cm\(^{-1}\) for CMP, 12,160 M\(^{-1}\) cm\(^{-1}\) for CMP, 8700 M\(^{-1}\) cm\(^{-1}\) for TMP\(^{-}\) and 5000 M\(^{-1}\) cm\(^{-1}\) for Cy3 (Glen Research). Duplex DNA substrates were prepared by mixing equimolar concentrations (usually 3 μM) of the appropriate DNA strands in reaction buffer, which was subsequently heated at 90 °C for 5 min followed by slow cooling to 25 °C. Reference DNA I (Fig. 1a) was formed from strands 1 and 2 (Fig. 1c); reference DNA II was formed from strands 3 and 4; reference DNA III was formed from strands 5 and 6; competitor DNA series IV was formed from strands 7 and 8; competitor DNA series V was formed from strands 9 and 10; and competitor DNA series VI was formed from strands 11 and 12. The sequences of the oligodeoxynucleotides used in this study are given in Fig. 1c.

### Fluorescence titrations

Fluorescence titrations were performed as described,\(^{37}\) using a PTIQM-4 fluorimeter (Photon Technology International, Lawrenceville, NJ) equipped with a 75 W Xe lamp. All slit-widths were set at 0.5 mm. The temperature of sample in the 10 mm pathlength type 3 quartz fluorimeter cuvette (3 mL) (NSG Precision Cells Inc., Farmingdale, NY) was controlled using a Lauda RM6 recir-
culation waterbath (Brinkmann, Westbury, NY). Stirring was maintained throughout each experiment using a P-73 cylindrical cell stirrer with a diameter of 8 mm (NSG Precision Cells Inc., Farmingdale, NY). The corrected Cy3 fluorescence intensity (Fcorr) after the ith addition of protein and the initial corrected Cy3 fluorescence of the reference DNA (F0corr) were obtained as described. The average number of protein molecules bound per DNA molecule is given by:

\[ F_{\text{corr}}^i = (F_i - F_0) \frac{V_i}{V_0} \]  

where \( F_i \) is the fluorescence intensity after the ith addition of titrant; \( F_0 \) is the background fluorescence of the buffer, which is always negligible; \( V_i \) is the volume after the ith addition; and \( V_0 \) is the volume before the first addition.

The observed relative fluorescence change (\( \Delta F_{\text{obs}} \)) is defined as:

\[ \Delta F_{\text{obs}} = \frac{F_{\text{corr}}^i - F_{\text{corr}}^0}{F_{\text{corr}}^0} \]  

\( \Delta F_{\text{obs}} \) reaches its maximum value (\( \Delta F_{\text{max}} \)) when both ends of the reference DNA are bound with protein. Hence \( \Delta F_{\text{obs}} / \Delta F_{\text{max}} \) (\( 0 \leq \Delta F_{\text{obs}} / \Delta F_{\text{max}} \leq 1 \)) equals the average of protein molecules bound per DNA end, and thus the average number of protein molecules bound per DNA molecule is given by (2\( \Delta F_{\text{obs}} / \Delta F_{\text{max}} \)).

Salt-back titrations

Salt-back titrations were performed after completion of a regular titration; i.e., after all additions of RecBC have been made. A buffer containing the same components as the reaction buffer but with 4 M NaCl was titrated into the cuvettes and fluorescence measurements were made as described above. Data from the salt-back titration experiments were analyzed using the same model as that used to describe the binding of RecBC to the ends of reference DNA, which has been described in detail. In this model, RecBC (referred to as \( B \)) binds to each end of reference DNA (referred to as \( D \)) with the same binding constant, \( K_B \), because the reference DNA has nearly identical ends. The binding polynomial for this model, which has two independent and identical sites, is given as:

\[ P = 1 + 2K_{BB}B + K_B^2B^2 \]  

where \( B_f \) is the free concentration of protein.

The average number of protein molecules bound per DNA molecule is given by:

\[ \frac{B_{\text{bound}}}{D_T} = \frac{2K_BB}{1 + K_BB} = \Delta F_{\text{obs}} \Delta F_{\text{max}} \]  

where \( B_{\text{bound}} = [DB] + 2[B_2D] \), [DB] is the concentration of D with only one of its ends bound by B and [B_2D] is the concentration of D with both of its ends bound by B.

As described, \( \Delta F_{\text{obs}} / \Delta F_{\text{max}} \) can be expressed explicitly in terms of total protein concentration (\( B_T \)), total reference DNA concentration (\( D_T \)) and \( K_R \) as:

\[ \Delta F_{\text{obs}} = \frac{1 + K_B(B_T + 2D_T) - \sqrt{4K_BB + (1 - K_BB + 2K_DD)^2}}{4K_DD} \]  

Experimental fluorescence titrations, plotted as \( \Delta F_{\text{obs}} \) versus [\( B_T \)], were obtained at three different reference concentrations of DNA, \( D_T \), and analyzed by global non-linear least-squares (NLLS) analysis using Eq. (5) to obtain the best fit values of \( K_B \) and \( \Delta F_{\text{max}} \).

To calculate \( K_B \) at each [NaCl] during a salt-back titration, Eq. (5) is rearranged and \( \Delta F_{\text{obs}} \) is substituted using Eq. (2) to become Eq. (6):

\[ K_B = \frac{\Delta F_{\text{obs}} \Delta F_{\text{max}}}{\Delta F_{\text{obs}} \Delta F_{\text{max}} - 1} \left( 2D_T \frac{\Delta F_{\text{obs}} \Delta F_{\text{max}} - B_T}{\Delta F_{\text{max}}} \right) \]  

where \( \Delta F_{\text{obs}} \Delta F_{\text{max}} \) is the relative fluorescence change observed after each addition of NaCl, and \( \Delta F_{\text{max}} \) is determined previously from an independent titration experiment of reference DNA with RecBC.

**Conclusion:**

Equilibrium constants for RecBC binding to non-fluorescent DNA molecules (N) were obtained from analysis of competition binding studies. The analysis of competition data has been described, and the same analysis is used here. Briefly, three separate titration experiments were performed at three different concentrations of non-fluorescent competitor DNA (\( N_1, N_2 \) and \( N_3 \)). In each titration experiment, a constant concentration of competitor DNA (\( N_1, N_2 \) or \( N_3 \)) was added to a cuvette containing a Cy3-labeled reference DNA at 20 nM and then titrated with protein. Since the competitor DNA molecules used here have nearly identical ends (DNA N1 and V1), B should bind to both ends of N with binding constant \( K_B \). Then \( B_N \) and \( B_T \) can be related to the total concentration of non-fluorescent DNA (N1N), \( K_C, K_R \) and \( D_T \) as shown:

\[ B_T = B_0 \left( 1 + 2 \left( K_B N_1 + K_{BB} B_t \right)^2 + K_{BB} B_t \right) \]

\[ + 2B_0 \left( K_{BB} N_1 \left( \frac{K_B N_1 + K_{BB} B_t}{1 + K_{BB} B_t} \right) + \frac{K_{BB} B_t}{1 + K_{BB} B_t} \right) \]

Data from the three titration experiments at competitor DNA concentrations \( N_1, N_2 \) and \( N_3 \) were analyzed simultaneously using Eqs. (4) and (7), and the “implicit fitting” NLLS algorithm in Scientist (Micromath, St Louis, MO) without the need to obtain an explicit expression for \( B_T \). The values of \( K_B \) was allowed to float in this analysis, while the values of \( K_R \) and \( \Delta F_{\text{max}} \) were fixed at values determined from the analysis of independent titrations with reference DNA in the absence of competitor. The uncertainties for the independently determined values of \( K_B \) and \( \Delta F_{\text{max}} \) were propagated into the reported uncertainties in \( K_C \).

**Equilibrium binding of Mg\(^{2+}\) to RecBC and RecBC–reference DNA complex**

The MgCl\(_2\) titration data were analyzed by assuming there is one Mg\(^{2+}\)-binding site on RecBC as well as RecBC bound at one end of the reference DNA, and no significant binding of Mg\(^{2+}\) to the reference DNA. This model is sufficient in describing the data (Fig. 5a) and therefore we did not consider more complicated models involving more than one Mg\(^{2+}\)-binding site per RecBC. This one-Mg\(^{2+}\)-site model is represented in Scheme 1, where \( K_{B,\text{tot}} \) and \( K_I \) correspond to the total concentration of Mg\(^{2+}\)-bound RecBC and the binding constant of Mg\(^{2+}\)-bound RecBC to the reference DNA, respectively.
are the stepwise macroscopic binding constants for forming BD (D with only one of its ends bound by B) in the absence and in the presence of MgCl₂, respectively, while $K_{1,\text{no Mg}}$ and $K_2$ are the stepwise macroscopic binding constants for forming B-D (D with both of its ends bound by B) in the absence and in the presence of MgCl₂, respectively. The equilibrium constant of B binding to D in the presence of Mg²⁺ ($K_3$) is related to $K_1$ and $K_2$ as:

$$K_1 = 2K_R$$

Similarly, $K_{1,\text{no Mg}} = 2K_{R,\text{no Mg}}$ and $K_{2,\text{no Mg}}$ can be expressed in terms of equilibrium constant for B binding to D in the absence of Mg²⁺ ($K_{R,\text{no Mg}}$) as given as:

$$K_{1,\text{no Mg}} = 2K_{R,\text{no Mg}}$$

$K_{2,\text{no Mg}}$ is the equilibrium constant for one molecule of B binding to one Mg²⁺, while $K_{B,\text{Mg}}$ is the equilibrium constant for one molecule of B bound at one end of D binding to one Mg²⁺. Expressions for $B_f$ and $D_f$ are given in Eqs. (10), (11) and (12) respectively:

$$B_f = B_i \left(1 + k_{B,\text{Mg}}^B B_i + 2 \left( K_R k_{B,\text{Mg}}^B + K_{R,\text{no Mg}} \right) \right)$$

$$D_f = D_i \left(1 + 2B_i \left( k_{B,\text{Mg}}^B M_{Bf} + K_{R,\text{no Mg}} \right) \right)$$

$$M_{f} = M_{i} \left(1 + k_{B,\text{Mg}}^B B_i + 2 k_{B,\text{Mg}}^B M_{Bf} D_f \right)$$

In our experiment, since all the reference DNA molecules were bound with two molecules of RecBC before addition of Mg²⁺, $[BD]=|BD||Mg²⁺|=0$ and $D_f=|B-D|+|B_2D|+|B|+|B|+|B_2D|+|B|$. Therefore Eqs. (10)–(12) become Eqs. (13)–(15):

$$B_f = B_i \left(1 + k_{B,\text{Mg}}^B M_{Bf} \right)$$

$$D_f = D_i \left(1 + k_{B,\text{Mg}}^B M_{Bf} \right)$$

$$M_{f} = M_{i} \left(1 + k_{B,\text{Mg}}^B B_i + 2 k_{B,\text{Mg}}^B M_{Bf} D_f \right)$$

By combining Eqs. (13) through (15), one obtains Eq. (16):

$$M_{f} = M_{i} \left(1 + k_{B,\text{Mg}}^B (B_f - 2D_f) \right) + 2 k_{B,\text{Mg}}^B D_f$$

which relates $M_{f}$ to $M_{i}$, $B_f$, $D_f$, $k_{B,\text{Mg}}^B$ and $k_{B,\text{Mg}}^B$. $\Delta F_{\text{obs},[Mg]}$ in this experiment reaches its maximum value ($\Delta F_{\text{max},[Mg]}$) when magnesium ions are bound at both ends of $B_2D$ to form $B_2D\bullet(Mg^{2+})_2$. Hence $\Delta F_{\text{obs},[Mg]} / \Delta F_{\text{max},[Mg]} (0|\Delta F_{\text{obs},[Mg]} / \Delta F_{\text{max},[Mg]}|\leq 1)$ equals the average number of magnesium ions bound per RecBC-bound DNA end. The average number of magnesium ions bound per RecBC-saturated DNA molecule is given by:

$$\frac{M_{\text{bound}}}{D_f} = \frac{2k_{B,\text{Mg}}^B M_{Bf}}{1 + k_{B,\text{Mg}}^B M_{Bf}} = \frac{2 \Delta F_{\text{obs},[Mg]} / \Delta F_{\text{max},[Mg]} + 2 \Delta F_{\text{corr},[Mg]} / \Delta F_{\text{max},[Mg]} - 2 \Delta F_{\text{corr},[Mg]} / \Delta F_{\text{max},[Mg]}}{2 \Delta F_{\text{corr},[Mg]} / \Delta F_{\text{max},[Mg]} - 2 \Delta F_{\text{corr},[Mg]} / \Delta F_{\text{max},[Mg]}}$$

where $M_{\text{bound}} = 2(|B_2D\bullet(Mg^{2+})_2| + |B_2D\bullet(Mg^{2+})_2|)$. $F_{\text{corr},[Mg]}$ is the corrected fluorescence intensity after the $i$th addition of Mg²⁺, $F_{\text{corr}}$ is the corrected fluorescence intensity before the addition of Mg²⁺, and $F_{\text{max},[Mg]}$ is the maximum value reached by $F_{\text{corr}}[Mg]$ after magnesium ions are bound at both ends of $B_2D$. Data were analyzed using Eqs. (16) and (17) and the implicit fitting NLLS algorithm in Scientist (Micromath, St. Louis, MO) without the need to obtain an explicit expression for $Mg$. In this analysis, the values of $k_{B,\text{Mg}}^B$, $k_{B,\text{Mg}}^B$, and $F_{\text{max},[Mg]}$ were allowed to float. All uncertainties are reported at the 68% confidence limit (± one standard deviation).

### Isothermal titration calorimetry

ITC experiments were performed with a VP-ITC calorimeter (Microcal, Northampton, MA) as described. The analysis of the calorimetric data has been described in detail, and the same analysis is used here. Briefly, experiments were carried out by titrating RecBC (0.7–1.1 μM in the sample cell) with 10 μL aliquots of DNA (8–14 μM in the syringe) at intervals of 4 min and at a stirring rate of 140 rpm. All samples were degassed before use. The heat of reaction was obtained by integration of the peak obtained after each injection by injecting the same volumes of DNA into the sample cell containing only buffer. The observed heat for the $i$th injection ($\Delta Q_i$) was obtained after correcting for the heat of dilution as described, and is related to the total heat after the $i$th injection ($Q_{tot}$) as in:

$$\Delta Q_i = Q_{tot} - Q_{tot,i+1} = \frac{dV_i}{V_0} (Q_{tot} - Q_{tot,i+1})$$

where $dV_i$ is the volume of the $i$th injection and $V_0$ is the active cell volume (1.43 mL). Since the DNA molecules used here have nearly identical ends (DNA VI series in Fig. 1a), the same model of two identical and independent sites (see Eq. (7)) was used to analyze $Q_{tot}'$, as given by:

$$Q_{tot}' = \Delta H_{\text{obs}} V_0 D_f \frac{2k_{B}^B}{1 + k_{B}^B} \frac{1 + k_{N}^B}{k_{B}^B}$$

$$\times \left(1 + k_{N}^B (B_f + 2D_f) - \sqrt{4k_{B}^B B_f + (1 - k_{N}^B B_f + 2k_{N}^B D_f)^2} \right)$$

where $\Delta H_{\text{obs}}$ is the observed enthalpy change for RecBC binding to one end of DNA, $D_f$ is the total DNA concentration in the cell after the $i$th injection, $K_N$ is the binding constant for RecBC binding to one DNA end, and $B_f$ and $D_f$ are the concentrations of free and total RecBC, respectively, in the cell after the $i$th injection. $\Delta H_{\text{obs}}$ and $K_N$ were obtained from NLLS analysis using Eqs. (18) and (19), and the ITC NLLS algorithm contained within the Origin 7.0 software as described.
In Fig. 6a and b, the observed heat released upon the ith injection normalized to the amount of injected DNA \( (\Delta Q_{\text{norm}}) \) is obtained using:

\[
\Delta Q_{\text{norm}} = \frac{\Delta Q_i}{dVdT^i}
\]  

where \( D^i \) is the concentration of DNA in the syringe. The continuous lines in Fig. 6a and b are simulations based on Eqs. (18)–(20) and the best fit values of \( \Delta H_{\text{obs}} \) (Table 4) and \( K_{\text{BC}} \) indicated in the figure legends.

The observed heat capacity change \( (\Delta C_p)_{\text{obs}} \) was obtained from a linear regression of \( \Delta H_{\text{obs}} \) obtained at different temperature using:

\[
\Delta H_{\text{obs}} = \Delta H_{\text{obs,ref}} + \Delta C_p(T - T_{\text{ref}})
\]

where \( \Delta H_{\text{obs,ref}} \) is the observed enthalpy change at some reference temperature \( (T_{\text{ref}}) \). The dependence of \( K_{\text{BC}} \) on temperature is described by the van’t Hoff equation

\[
\frac{\Delta H_{\text{obs,ref}}}{R} = -\Delta H_{\text{BC},0}/R
\]

where \( R \) is the gas constant.

### Appendix A

The probability that a nucleotide within a duplex DNA is bound with only Na\(^+\) and no Mg\(^{2+}\) when the duplex DNA is placed in a buffer containing both cations is calculated using Eq. (A1):

\[
P_{\text{Na}} = \frac{[D_0]}{[D]} = \frac{2}{1 + \sqrt{1 + 4K_{\text{obs}}^Mg^2+[Mg^{2+}]}}
\]

where \([D]\) is the total nucleotide concentration, \([D_0]\) is the concentration of nucleotides associated with only Na\(^+\) and \( K_{\text{obs}}^Mg \) is the observed intrinsic constant for Mg\(^{2+}\) binding to each DNA site. As shown by Eq. (A1), \( P_{\text{Na}} \) equals 1 before any addition of Mg\(^{2+}\) and it will decrease as [Mg\(^{2+}\)] increases, indicating an increasing probability of Mg\(^{2+}\) binding to the DNA.

The dependence of \( K_{\text{obs}}^Mg \) on [NaCl] has been determined from the non-specific interactions between lac repressor and duplex DNA, as well as pentasyne and duplex DNA, and is given in Eqs. (A2) and (A3), respectively:

\[
\log K_{\text{obs}}^Mg = -(1.7 \pm 0.1) \log [\text{NaCl}] + (0.3 \pm 0.2)
\]

In the presence of 100 mM NaCl, using either Eq. (A2) or Eq. (A3), one obtains an estimate for \( K_{\text{obs}}^Mg \approx 100 \text{ M}^{-1} \). Thus, in the presence of 10 mM MgCl\(_2\) and 100 mM NaCl one obtains a value of \( P_{\text{Na}} \approx 0.62 \) using Eq. (A1). Similarly, in the presence of 400 mM NaCl, \( K_{\text{obs}}^Mg = 9.5 \text{ M}^{-1} \) and \( P_{\text{Na}} \approx 0.95 \) when 10 mM MgCl\(_2\) is present.

### References


8. Veau, X., Delmas, S., Selva, M., Jeusset, J., Le Cam,


