Interaction of Rep and DnaB on DNA

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ABSTRACT

Genome duplication requires not only unwinding of the template but also the displacement of proteins bound to the template, a function performed by replicative helicases located at the fork. However, accessory helicases are also needed since the replicative helicase stalls occasionally at nucleoprotein complexes. In *Escherichia coli*, the primary and accessory helicases DnaB and Rep translocate along the lagging and leading strand templates, respectively, interact physically and also display cooperativity in the unwinding of model forked DNA substrates. We demonstrate here that this cooperativity is displayed only by Rep and not by other tested helicases. ssDNA must be exposed on the leading strand template to elicit this cooperativity, indicating that forks blocked at protein–DNA complexes contain ssDNA ahead of the leading strand polymerase. However, stable Rep–DnaB complexes can form on linear as well as branched DNA, indicating that Rep has the capacity to interact with ssDNA on either the leading or the lagging strand template at forks. Inhibition of Rep binding to the lagging strand template by competition with SSB might therefore be critical in targeting accessory helicases to the leading strand template, indicating an important role for replisome architecture in promoting accessory helicase function at blocked replisomes.

INTRODUCTION

The complex multi-subunit machines that replicate chromosomal DNA possess high processivity but many blocks to replication exist that necessitate removal or bypass of the block for successful completion of genome duplication. Damage to the template DNA presents one such obstacle but proteins bound to the template also pose barriers to replisome movement (1). In particular, transcribing or stalled RNA polymerases are a class of abundant, high affinity nucleoprotein complexes that present significant challenges to completion of genome duplication (2–6). Recombination enzymes play important roles in the repair and restart of such blocked replication forks but the recruitment of recombination is associated with the risk of unintended genome rearrangements (7–11). A non-recombinogenic alternative to resuscitating forks blocked by nucleoprotein complexes is the recruitment of additional helicases/ translocases to such blocked forks. Such motors can translocate along DNA and actively displace proteins from the nucleic acid (12), with displacement being promoted by translocation of multiple helicases along the DNA (13). Thus if the primary replicative helicase is blocked by a nucleoprotein complex, the ability to recruit multiple accessory helicases to the fork may facilitate clearance of such blocks.

Rep may provide such an accessory replicative helicase in *Escherichia coli* as movement of replication forks through model nucleoprotein blocks is promoted by this helicase both *in vitro* and *in vivo* (14,15). A second Superfamily 1 helicase in *E. coli*, UvrD, also promotes fork movement through protein–DNA complexes suggesting that Rep and UvrD may both provide accessory replicative helicase activity in a redundant manner (14,15). In support of such a redundancy, although cells lacking either Rep or UvrD are viable, the absence of both is lethal under rapid growth conditions (14–16). A third helicase, DinG, is also required for efficient duplication of highly transcribed *rrn* operons where the direction of transcription opposes the direction of fork movement (15).

Three helicases might therefore underpin fork movement along protein-bound DNA in *E. coli*. However, mean replication fork speed is reduced in cells lacking Rep but not in cells lacking either UvrD or DinG (17,18). Furthermore, Rep but not UvrD interacts physically with the primary replicative helicase DnaB, an interaction that facilitates Rep accessory helicase activity both *in vitro* and *in vivo* (14,18). These data suggest that Rep rather than UvrD or DinG may provide the primary means of underpinning replication fork movement along protein-bound DNA in wild-type cells.

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Rep translocates from 3′ to 5′ along single-stranded DNA (19) whereas the hexameric DnaB helicase translocates from 5′ to 3′ along ssDNA (20). On model forked DNA substrates *in vitro* Rep and DnaB display cooperativity in unwinding such substrates, a cooperativity that correlates with the formation of stable Rep–DnaB-forked DNA complexes (14). In contrast, UvrD does not form stable complexes with DnaB and forked DNA and does not display cooperativity with DnaB in unwinding DNA (14). These data again support Rep being the accessory helicase in wild-type *E. coli* cells. Given the opposing polarities of Rep and DnaB, these data are also consistent with accessory helicase activity being directed to the leading strand template whilst the primary replicative helicase translocates along the lagging strand template (14). Complex formation between Rep and DnaB at forked DNA structures may therefore be critical for Rep function during promotion of replisome movement. However, the impact of the structure of the DNA substrate on the interaction between Rep and DnaB is unknown.

We have analysed the physical and functional interactions between Rep and DnaB on a range of DNA structures. We find that ssDNA must be present on the leading strand template to observe cooperative unwinding of forked DNA structures by Rep and DnaB, with apparent cooperativity increasing with the extent of ssDNA exposed on the leading strand template. These data imply that ssDNA must be present on the template ahead of the leading strand polymerase at blocked replication forks. However, formation of stable Rep/DnaB/DNA complexes does not require branched DNA as such complexes can form with linear ssDNA of >30 bases. Thus Rep–DnaB complex formation is not DNA structure-specific. Productive interaction of Rep with DnaB on DNA substrates is dictated therefore solely by the opposing polarities of these two helicases in combination with the availability of ssDNA with opposing polarities at replication forks.

MATERIALS AND METHODS

Proteins and DNA substrates

Rep and DnaB were purified as described (21,22). RepK28A was purified in the same manner as wild-type protein using the same overexpression plasmid as wild-type *rep* except that codon 28, AAA, had been mutated to GCA. *Bacillus stearothermophilus* PcrA, *E. coli* DinG and *Deinococcus radiodurans* RecD2 were kind gifts of Panos Soultanas (University of Nottingham, UK), Rafael Daniel Camerini-Otero (NIDDK, National Institutes Of Health, Bethesda, USA) and Dale Wigley (Cancer Research UK), respectively. Single-stranded oligonucleotides were labelled at the 5′ end using [γ^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs) before passage through Microbiospin 6 columns (BioRad) to remove unincorporated label. DNA substrates containing more than one strand were constructed by annealing of oligonucleotides, one of which was labelled, and purification by gel electrophoresis (23). Sequences of oligonucleotides other than (dT)n are shown in Table 1 whilst the oligonucleotide composition of multi-stranded DNA structures are indicated in Table 2. DNA concentrations refer to the concentration of the DNA structure rather than nucleotide equivalents.

DNA-binding assays

DNA binding was monitored using a gel electrophoretic mobility shift assay. Proteins at the indicated concentrations were added to 1 nM of labelled DNA substrate that had been preincubated for 2 min at 37°C in 50 mM HEPES pH 8.0, 10 mM magnesium acetate, 10 mM DTT, 10 μM ADP and 50 μg ml^{-1} bovine serum albumin. The final reaction volume was 10 μl. Where applicable, DnaB was added prior to Rep. Incubation was continued for 10 min at 37°C prior to addition of 2 μl of 30% glycerol. Reactions were then loaded onto a 4% polyacrylamide gel in 89 mM Tris base, 89 mM boric acid, 10 mM magnesium acetate and 10 μM ADP with

Table 1. Oligonucleotide sequences used in construction of DNA substrates

Oligonucleotide	Sequence
1	GCAAGCCTTCTACAGGTCGACCGTCCATGGCGACTCGAGACCGCAATACGGATAAGGGCTGACTATCTACGTCCGAGGCTCGCGCCGCAGACTCATTT
2	GAGCACGCCGACGAACATTCACCACGCCAGACCACGTAAGCCCTTATCCGTATTGCGGTCTCGAGTCGCCATGGACGGTCGACCTGTAGAAGGCTTGC
3	AAATGAGTCTGCGGCGCGAGCCTCGGACGTAGATAGTC
4	TACGTGGTCTGGCGTGGTGAATGTTCGTCGGCGTGCTC
5	AGCCCTTATCCGTATTGCGGTCTCGAGTCGCCATGGACGGTCGACCTGTAGAAGGCTTGC
6	GCAAGCCTTCTACAGGTCGACCGTCCATGGCGACTCGAGACCGCAATACGGATAAGGGCT
7	GCAAGCCTTCTACAGGTCGACCGTCCATGGCGACTCGAGACCGCAATACGGATAAGGGCTTTTTTTGACT—ATCTACGTCCGAGGCTCGCGCCGCAGACTCATTT
8	GCAAGCCTTCTACAGGTCGACCGTCCATGGCGACTCGAGACCGCAATACGGATAAGGGCTTTTTTTTTTTTT—TGACTATCTACGTCCGAGGCTCGCGCCGCAGACTCATTT
9	GCAAGCCTTCTACAGGTCGACCGTCCATGGCGACTCGAGACCGCAATACGGATAAGGGCTTTTTTTTTTTTTTTTTTGACTATCTACGTCCGAGGCTCGCGCCGCAGACTCATTT
10	GCAAGCCTTCTACAGGTCGACCGTCCATGGCGACTCGAGACCGCAATACGGATAAGGGCTGAGCACGCCGACGAACATTCACCACGCCAGACCACGTA
11	GACTATCTACGTCCGAGGCTCGCGCCGCAGACTCATTTAGCCCTTATCCGTATTGCGGTCTCGAGTCGCCATGGACGGTCGACCTGTAGAAGGCTTGC

All oligonucleotides are written 5′–3′.

Table 2. Oligonucleotide composition of DNA structures used in this study

Substrate number	Oligonucleotide numbers
1	1 + 2*
2	1 + 2* + 3
3	1 + 2* + 4
4	1 + 2* + 3 + 4
5	2* + 3 + 7
6	2* + 3 + 8
7	2* + 3 + 9
8	1* + 5
9	2 + 6*
10	10 + 11*
11	10 + 11* + 4
12	10 + 11* + 3
13	10 + 11* + 3 + 4

Oligonucleotide numbers refer to Table 1.
Asterisks indicate the 5′-labelled oligonucleotide in each substrate.

electrophoresis being performed at 160 V for 90 min at room temperature before the gel was dried and analysed by autoradiography and phosphorimaging.

Fork unwinding assays

Reactions containing 1 nM DNA substrate in 50 mM HEPES pH 8.0, 10 mM magnesium acetate, 10 mM DTT, 2 mM ATP and 200 µg ml^{-1} bovine serum albumin were assembled on ice and then incubated at 37°C for 2 min. DnaB was added where indicated and incubation continued for another 2 min at 37°C. Rep was then added where indicated to give a final reaction volume of 10 µl. Incubation was then continued for 10 min at 37°C before terminating the reactions by addition of 2.5 µl of 100 mM Tris–HCl pH 7.5, 200 mM EDTA, 10 mg ml^{-1} proteinase K and 0.5% SDS followed by 2.5 µl of 30% glycerol plus 0.25% bromophenol blue. Reactions were then analysed by non-denaturing electrophoresis (24).

RESULTS

Cooperativity between Rep and DnaB in unwinding forked DNA requires single-stranded DNA on both arms of the fork

Physical interaction between DnaB and Rep results in stable formation of DnaB–Rep–DNA complexes with forked DNA substrates bearing two ssDNA arms (14). Formation of this complex correlates with enhanced levels of unwinding of the DNA substrate when both helicases are present as compared with the sum of activities for each individual helicase (14). Given that DnaB and Rep have opposing polarities of translocation along ssDNA (19,20), these data suggest that Rep and DnaB cooperate in unwinding such substrates by binding of DnaB to the ssDNA equivalent to the lagging strand template in model forked DNA substrates and binding of Rep to the ssDNA equivalent to the leading

strand template. We tested this model directly by analysing unwinding of forked DNA substrates with and without ssDNA on the leading and lagging strand templates.

As shown previously (14), with a forked DNA possessing two ssDNA strands at the fork, cooperativity in unwinding was observed with Rep plus DnaB relative to the sum of unwinding by individual helicases (Figure 1A, lanes 1–4; Figure 2A and C). Moreover, cooperativity was observed on this substrate regardless of the order of addition of Rep and DnaB (data not shown). The relative importance of ssDNA being present on the arm equivalent to the leading strand template was tested using substrate 2, bearing dsDNA rather than ssDNA on this arm. Unwinding of this substrate by Rep was inhibited as compared with substrate 1 (Figure 1A, compare lanes 2 and 6 and 1B), reflecting a requirement for this 3′–5′ ssDNA translocase to bind to and move along the leading strand template to effect unwinding of such substrates. In contrast, unwinding by DnaB alone resulted in a greater fraction of substrate 2 being unwound as compared with substrate 1 (Figure 1A, compare lanes 3 and 7 and 1B). Such stimulation by the presence of dsDNA as opposed to ssDNA on the leading strand arm is consistent with previous observations (25). This stimulation is likely due to the increased rigidity of this dsDNA arm reducing the probability of this arm being located within the central cavity of DnaB as it translocates along the lagging strand arm, a reaction that would not result in unwinding of the substrate (25). Upon addition of both Rep and DnaB to substrate 2, no major enhancement of unwinding was observed as compared with each individual helicase (Figure 1A, compare lanes 6–8 and 1B). This lack of enhancement of unwinding of substrate 2 was observed at all tested concentrations of Rep (Figure 2B and C). The single-stranded versus double-stranded nature of the leading strand arm has therefore a major impact on cooperative unwinding of branched DNA by Rep and DnaB.

Unwinding of a fork bearing a dsDNA lagging strand arm was also analysed. Unwinding by DnaB was inhibited as compared with substrates 1 and 2 (Figure 1A, compare lanes 3, 7 and 11), as expected given the requirement of this 5′-3′ translocase to bind to and translocate along the lagging strand arm. Unwinding of substrate 3 by Rep was also inhibited as compared with substrates 1 and 2 (Figure 1A, compare lanes 2, 6 and 10). The cause of this inhibition is unclear since translocation of Rep 3′–5′ along the leading strand arm should not have been inhibited by the duplex lagging strand arm. One possible explanation is that a higher binding affinity of Rep for the ssDNA on the lagging strand arm as compared with ssDNA on the leading strand arm (Figure 6A, compare lanes 6 and 10) might have increased the local concentration of Rep at the fork in substrate 1 as compared with 3, enhancing Rep-catalysed unwinding of substrate 1. However, this point was not explored further. Regardless of the cause of this reduced unwinding by Rep, the presence of both Rep and DnaB did not result in any enhancement of unwinding of this substrate (Figure 1A, lanes 10–12 and 1B). Similarly, no
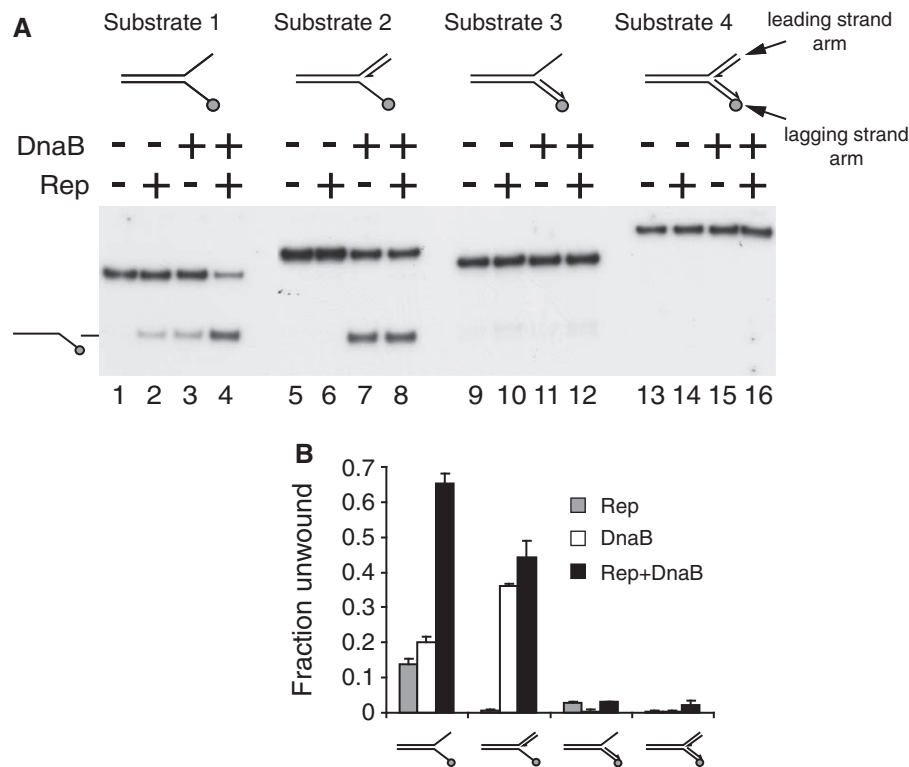
Figure 1. Cooperativity between DnaB and Rep in unwinding of forked DNA structures requires ssDNA on both leading and lagging strand arms. (**A**) Unwinding of substrates 1, 2, 3 and 4 by the indicated combinations of Rep and DnaB. (**B**) The fraction of substrates 1–4 unwound by the indicated combinations of Rep and DnaB. The concentration of Rep was 10 nM and the concentration of hexameric DnaB was 10 nM. Shaded circles indicate the positions of the radiolabel whilst the arrow indicates the 3′ end of oligonucleotides.

cooperativity of unwinding was observed when both leading and lagging strand arms were double-stranded (Figure 1A, lanes 13–16) as expected.

To exclude the possibility of DNA sequence-dependent secondary structures modulating Rep- and DnaB-catalysed unwinding of substrates 1–4 the unwinding of a second set of branched substrates was analysed. The sequences of the leading and lagging strand arms of this second set of substrates were reversed with respect to substrates 1–4. Cooperative unwinding by Rep and DnaB was observed on the forked DNA having two ssDNA arms, as seen with substrates 1–4 (Supplementary Figure S1). Unwinding of the forked DNA having a duplex leading strand arm and a single-stranded lagging strand arm was also enhanced in the presence of both Rep and DnaB but this enhancement was small as compared with the fork bearing two ssDNA arms (Supplementary Figure S1B, compare substrate 11 with 10). We conclude that the presence of ssDNA on both the leading and lagging strand arms of forked DNA is required to observe significant levels of cooperativity between Rep and DnaB.

Rep can promote movement of replisomes that have become blocked by nucleoprotein complexes but otherwise retain the ability to resume replication upon clearance of the block (14). A requirement for ssDNA to be present on the leading strand template implies therefore that such ssDNA is accessible even within the context of an active replisome. We probed therefore the length of

ssDNA on the leading strand template required to observe cooperativity between Rep and DnaB. A series of substrates were constructed that differed only in the length of the ssDNA present on the leading strand template at the branch point of the fork. The degree of cooperativity increased as the length of this ssDNA on the leading strand template increased from 0 to 18 nt (Figure 3). Increased cooperativity was observed even with only 6 nt on the leading strand template. Given the 8-nt binding site size of Rep, as judged by X-ray crystallography (26), these data imply that initial access of even a single Rep monomer may be sufficient to facilitate cooperative unwinding of DNA in conjunction with DnaB.

One model to explain the observed cooperativity between Rep and DnaB is that physical coupling of two active helicases at a fork increases the probability of substrate unwinding. Alternatively, binding of one motor to the fork might act merely as a platform for the second motor, increasing the local concentration of the second motor at the fork. We tested this possibility by using a mutant Rep in which the invariant lysine within helicase motif I, known to be essential for ATP hydrolysis and hence helicase activity (26–30), was replaced by alanine. Absence of cooperativity between RepK28A and DnaB indicated that Rep helicase activity was essential for enhancement of unwinding with DnaB (Figure 4). This lack of cooperativity was reflected in the inability of RepK28A to promote *E. coli* replication fork movement through
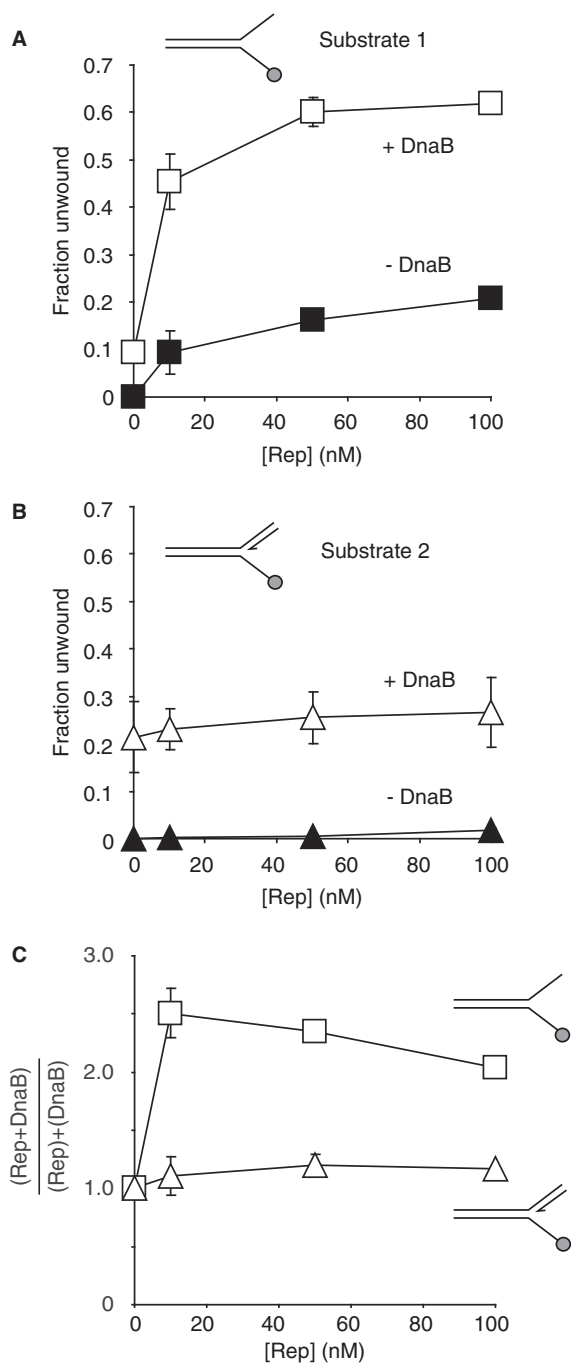
Figure 2. Cooperativity in unwinding by DnaB and Rep is observed on forked DNA bearing a ssDNA leading strand arm but not a dsDNA leading strand arm. (**A**) and (**B**) Unwinding of substrates 1 and 2 by the indicated concentrations of Rep in the absence and in the presence of 10 nM DnaB hexamers. (**C**) Relative levels of substrate unwinding by Rep plus DnaB in comparison to the sum of unwinding by each individual helicase.

Figure 3. Cooperativity between DnaB and Rep increases with the size of ssDNA exposed on the leading strand template at forks. Unwinding of substrates 2 and 5–7 was monitored in the presence of 10 nM Rep, 10 nM DnaB hexamers and 10 nM Rep +10 nM DnaB hexamers. The relative levels of unwinding by Rep plus DnaB in comparison to the sum of unwinding by each individual helicase is shown.

protein–DNA complexes *in vitro* (data not shown). We were unable to test whether binding of DnaB was sufficient to stimulate Rep-catalysed unwinding since mutation of the equivalent helicase motif I lysine in DnaB rendered the protein insoluble (data not shown). Howeve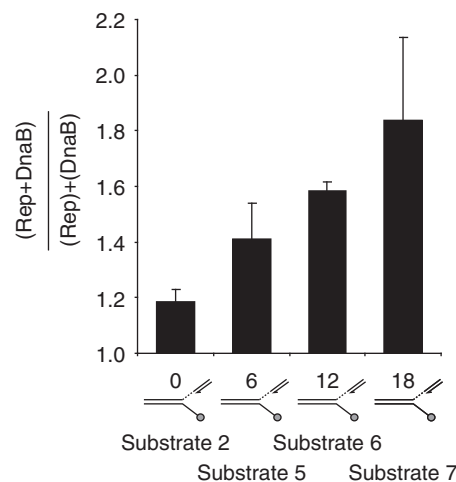r, these data do indicate that Rep does not play a merely structural role in stimulation of DnaB and promotion of replisome movement along protein-bound DNA.

Cooperativity is specific to Rep

Previous work indicated that, although both Rep and UvrD can promote replisome movement through nucleoprotein complexes, cooperativity with DnaB was exhibited only by Rep and not by UvrD (14). We also tested for cooperativity between *E. coli* DnaB and a 3′–5′ Superfamily 1 helicase from *B. stearothermophilus*, PcrA, that shares significant homology with UvrD and Rep and that can also promote movement of *E. coli* replisomes along protein-bound DNA (14). However, there was no detectable cooperativity between *E. coli* DnaB and *B. stearothermophilus* PcrA (Figure 5). This lack of enhancement in unwinding supports the conclusion that cooperativity between Rep and DnaB does not simply reflect a lowering of the processivity barrier by partial unwinding of the substrate by one motor and subsequent completion of unwinding by a second motor.

The ability of 5′-3′ helicases to elicit cooperativity with DnaB, as opposed to the 3′–5′ helicase Rep, was also tested. However, cooperativity with DnaB was not observed with *D. radiodurans* RecD2, a 5′–3′ Superfamily 1 helicase (31). *E. coli* DinG, a 5′–3′ Superfamily 2 helicase (32) that, like Rep and UvrD, promotes replication of transcribed DNA (15) also failed to give detectable enhancement of unwinding with DnaB (Figure 5). These data all support a model in which physical association of DnaB with a 3′–5′ helicase, Rep, enhances unwinding of branched DNA possessing ssDNA on both arms of the fork.
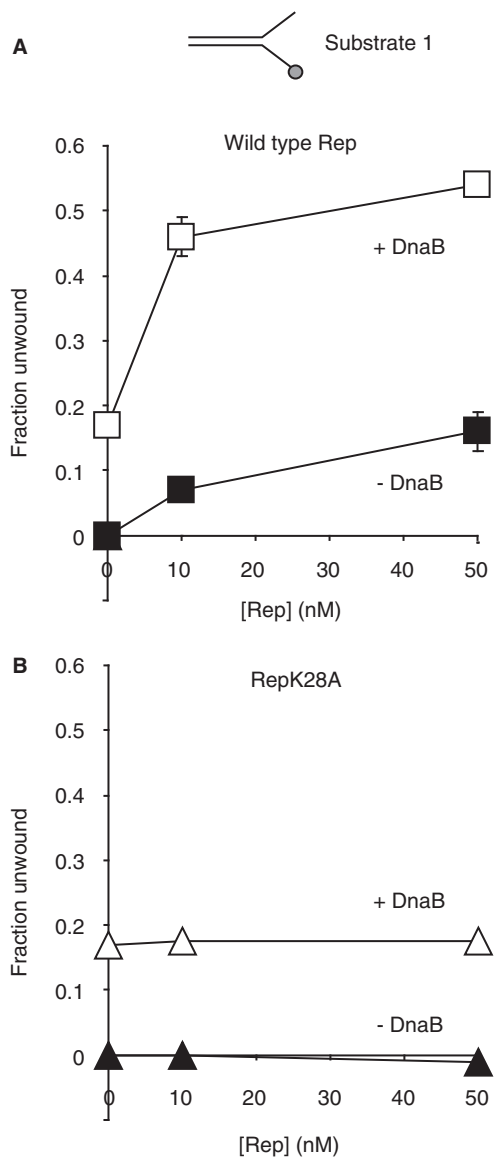
Figure 4. Binding of Rep is not sufficient to enhance DNA unwinding by DnaB. (**A**) Unwinding of substrate 1 by the indicated concentrations of wild-type Rep in the absence and in the presence of 10 nM DnaB hexamers. (**B**) Unwinding of substrate 1 by RepK28A without and with 10 nM DnaB hexamers.

Figure 5. Cooperativity with DnaB is specific to Rep. Unwinding of substrate 1 by 0, 10, 50 and 100 nM *E. coli* Rep and DinG, *B. stearothermophilus* PcrA or *D. radiodurans* RecD2 was monitored in the absence and in the presence of 10 nM *E. coli* DnaB hexamers. Relative levels of substrate unwinding by the test helicase plus DnaB in comparison to the sum of unwinding by the individual test helicase and DnaB is shown as a function of the concentration of the test helicase.

Cooperativity between Rep and DnaB helicase activities does not correlate with formation of Rep–DnaB–DNA complexes

Cooperativity between Rep and DnaB has been correlated with formation of stable Rep–DnaB–DNA complexes (14) and so we analysed the binding of DnaB and Rep to substrates 1–4. Rep and DnaB each bound substrate 1, although the Rep–substrate 1 complex was unstable as judged by the lack of a distinct Rep–DNA complex (Figure 6A, lanes 1–3). Upon addition of both helicases to substrate 1, a supershifted protein–DNA complex was observed indicative of a stable Rep–DnaB–DNA complex (Figure 6A, lane 4) regardless of the order of addition of proteins to the DNA (data not shown). Thus, as seen
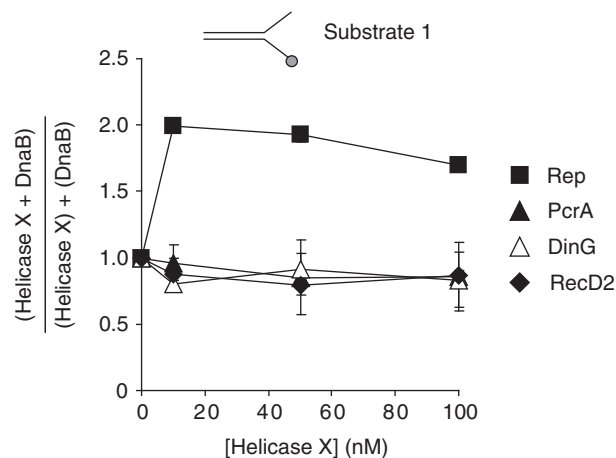
previously (14), enhancement of unwinding of a fork possessing two ssDNA arms by the presence of both Rep and DnaB is reflected in the formation of a stable Rep–DnaB–DNA complex. As expected, binding of Rep and DnaB required ssDNA within the substrate since a fork possessing duplex leading and lagging strand arms did not display any binding (Figure 6A, lanes 13–16). However, when binding to substrates 2 and 3 was analysed, although no supershifted complex was observed with substrate 3 (Figure 6A, lanes 9–12), such a complex was observed with substrate 2 (Figure 6A, lanes 5–8). Since cooperativity of unwinding was observed neither for substrate 2 nor substrate 3 (Figure 1A), cooperativity in DNA unwinding does not correlate therefore with stable Rep–DnaB–DNA complex formation.

The formation of a stable Rep–DnaB-substrate 2 complex indicates that both Rep and DnaB can bind to the 38 bases of ssDNA exposed on the equivalent of the lagging strand arm in the substrate. In other words, Rep and DnaB can form a stable tripartite complex with unbranched ssDNA. This was tested directly using two unbranched substrates equivalent to substrate 1 but lacking one or the other of the ssDNA arms. No stable binding of Rep and/or DnaB was observed with substrate 8 (Figure 6B, lanes 5–8) consonant with the lack of binding to the same ssDNA arm present in substrate 3 (Figure 6A, lanes 9–12). In contrast, a supershifted complex was observed in the presence of Rep and DnaB with substrate 9 reflecting the formation of a similar complex with substrate 2 bearing the same ssDNA arm (compare lanes 5–8 in Figure 6A with lanes 9–12 in 6B). These data suggest that branched ssDNA is not a requirement for formation of a Rep–DnaB–DNA complex. However, differences in binding of substrates 8 and 9 also indicate DNA sequence-dependent and possibly therefore DNA structure-dependent effects upon binding
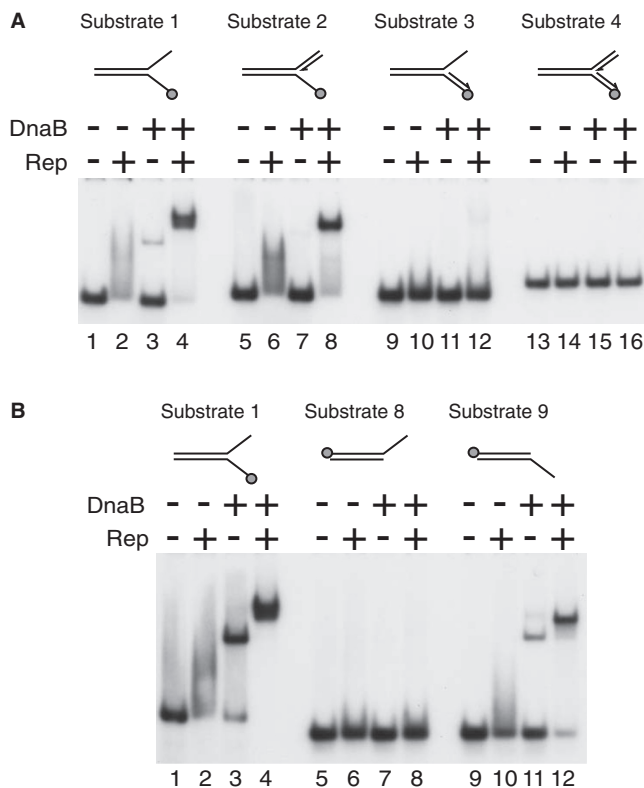
Figure 6. Formation of stable Rep–DnaB–DNA complexes does not require branched DNA. (**A**) gel mobility shift assays with substrates 1–4 in the presence of 10 nM Rep and 10 nM DnaB hexamers as indicated. (**B**) gel mobility shift assays of substrates 1, 8 and 9 with 10 nM Rep and 100 nM DnaB hexamers. Note that similar patterns were also observed with substrates 1, 8 and 9 with 10 nM Rep and 10 nM DnaB hexamers (data not shown).

Figure 7. Rep and DnaB can form stable complexes with linear ssDNA. (**A**) binding of dT_{60} by 10 nM Rep (lanes 2, 6–8) in the presence of 10, 25 and 100 nM DnaB hexamers as indicated. (**B**) binding of dT_{60}, dT_{50}, dT_{40} and dT_{30} by 10 nM Rep and 100 nM DnaB hexamers as indicated.

of Rep and DnaB. Binding of Rep and DnaB to homopolymeric ssDNA was analysed therefore. Both Rep and DnaB bound to dT_{60}, with Rep–DNA complexes again displaying low stability as judged by the absence of a defined complex in gels (Figure 7A, compare lane 2 with 3–5). In the presence of both Rep and DnaB, formation of a more slowly migrating species indicated a stable Rep–DnaB–dT_{60} complex (Figure 7A, compare lanes 3–5 with 6–8). These data confirm that Rep and DnaB can form a stable complex with unbranched ssDNA.

Analysis of Rep and DnaB binding to oligonucleotides of decreasing size demonstrated that supershifted complexes in the presence of both proteins were formed with 60- and 50-mer oligonucleotides (Figure 7B lanes 1–8). A supershifted complex was also formed with a 40-mer oligonucleotide, although the smeared nature of this complex after electrophoresis indicated reduced stability (Figure 7B, compare lane 12 with 4 and 8). Formation of a Rep–DnaB–ssDNA complex with oligonucleotides of 40 bases or greater is consistent with formation of such complexes with the 38 bases of ssDNA present in substrates 2 and 9 (Figure 6A, lanes 5–8; 6B, lanes 9–12). In contrast, there was little evidence of a stable Rep–DnaB–DNA complex with a 30-mer oligonucleotide (Figure 7B lanes 13–16). Taken together, these data demonstrate that formation of a stable
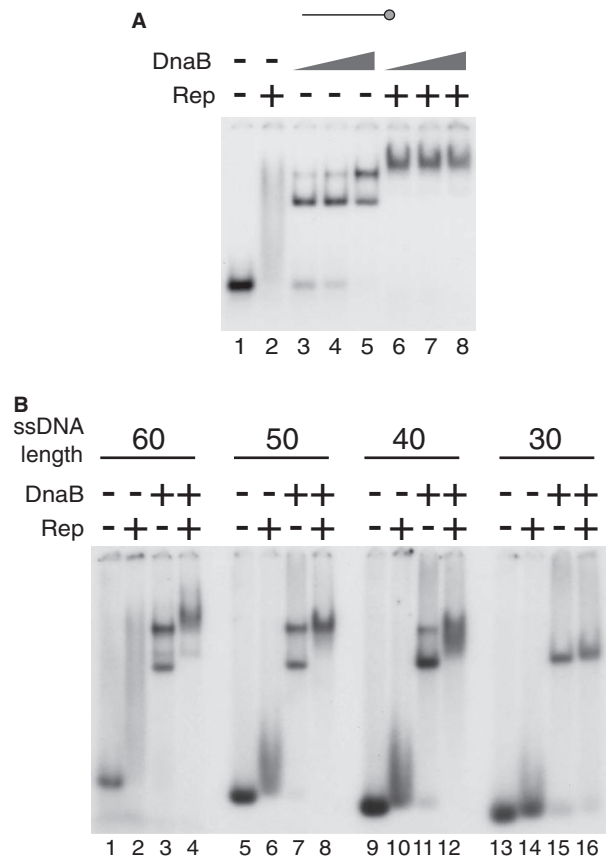
Rep–DnaB–ssDNA complex requires between 30 and 38 nt of unbranched single-stranded DNA.

DISCUSSION

The data presented here demonstrate that cooperative unwinding of forked DNA substrates in conjunction with *E. coli* DnaB is specific to Rep. This cooperativity requires ssDNA to be present on both arms of the branch, correlating with the 3′–5′ and 5′–3′ polarities of translocation of Rep and DnaB, respectively (19,20). The absence of significant cooperativity in unwinding forked DNA bearing a leading strand duplex and a single-stranded lagging strand arm (substrate 2) also indicates that translocation of DnaB along the lagging strand template to generate exposed ssDNA on the leading strand template is not an efficient means of promoting Rep–DnaB cooperativity, even at higher Rep concentrations (Figure 2B and C). Pre-existing single-stranded DNA on the leading strand template therefore facilitates cooperativity between Rep and DnaB.

Analysis of the size of ssDNA required on the leading strand template to promote cooperativity indicates that as little as six bases promotes functional interaction between Rep and DnaB (Figure 3). Since Rep makes significant

contacts with eight bases of ssDNA (26) our data indicate that initial binding of even a single Rep monomer on the leading strand template at forks may be sufficient to elicit cooperativity with DnaB. Interaction of a single Rep monomer with a DnaB hexamer might result therefore in cooperative unwinding of the DNA substrates used in this study. Such a possibility is in apparent contradiction of data indicating that wild-type Rep cannot act as a processive monomeric helicase *in vitro* (33,34). However, initial binding of a single Rep monomer at the branch point may be followed by binding of other Rep molecules behind the first after destabilization of a very limited number of base pairs, either by DnaB or by the Rep monomer. Alternatively, interaction of Rep with DnaB might result in activation of Rep monomer helicase activity. Although wild-type Rep monomers are not processive helicases, removal of the 2B subdomain results in monomers that can act as processive helicases, although processivity is still limited (34). This 2B subdomain can also undergo a large conformational rotation with respect to the other Rep subdomains, providing a possible explanation for activation/deactivation of Rep helicase activity (26). Interaction of Rep with DnaB might therefore result in alteration of the conformational state of the 2B subdomain and relief of inhibition of Rep monomer helicase activity. Such an activation mechanism might also explain the known ability of bacteriophage proteins to bind to and increase the processivity of Rep, allowing Rep to act as the replicative helicase during bacteriophage genome duplication (35–37). However, any conformational change in Rep induced by DnaB is unlikely to occur via a direct interaction between DnaB and the Rep 2B subdomain, given the absence of any detectable interaction between this subdomain and DnaB (14).

Regardless of the number of Rep monomers required for unwinding of forked DNA substrates in the presence of DnaB, it is clear that the formation of stable Rep–DnaB–DNA complexes is not specific to branched DNA (Figures 6B and 7). This lack of branched DNA specificity in terms of complex formation indicates that cooperation between Rep and DnaB at forked DNA structures is determined solely by the relative locations and polarities of the ssDNA-binding sites for Rep and DnaB at forks. Interaction between Rep and DnaB in terms of promoting fork progression along protein-bound DNA likely occurs within the context of blocked but still active replisomes. DnaB encircles the single-stranded DNA of the lagging strand template at or near the branch point of replication forks (38). A functional interaction between DnaB and Rep within such a context requires that Rep binds to and translocates along single-stranded DNA located on the leading strand template (Figures 1A and 2C). However, our data demonstrate that binding of Rep to the lagging strand template could also lead to formation of a stable Rep–DnaB–ssDNA complex that does not lead to cooperative unwinding (Figure 6A, lanes 5–8 and Figure 2C). Such a non-productive reaction might be inhibited *in vivo* by SSB binding to the extensive amount of ssDNA present on the lagging strand template. In contrast, any ssDNA present on the leading strand template ahead of the polymerase at the fork is unlikely to be extensive and so unlikely to be bound by SSB, given that SSB requires at least 35 nt of ssDNA for stable binding (39). Thus, whilst stable interaction of Rep and DnaB can occur even on linear ssDNA, competition with SSB may ensure that Rep activity is localized to ssDNA of the leading strand template exposed at the branch point of blocked forks. In effect, DNA structure specificity with respect to Rep–DnaB cooperativity may be conferred by the relative probabilities of binding of Rep and SSB to the leading and lagging strand templates.

One implication of such a specificity mechanism is that the exact means by which an accessory helicase might be localized to the replisome may not be critical. As long as the local concentration of the accessory helicase is sufficiently high and ssDNA is exposed on a template strand with the appropriate polarity then replisome movement along protein-bound DNA may be promoted. *Saccharomyces cerevisiae* Rrm3p, the only other known accessory replicative helicase (40), is associated with the replisome via interaction with the sliding clamp and possibly the leading strand polymerase rather than the replicative helicase (41,42). Interaction between the primary and accessory replicative helicases is not therefore a fundamental requirement for efficient duplication of protein-bound DNA. Co-localization of the accessory helicase with the replisome and the availability of a DNA-binding site with appropriate polarity, in contrast, may be critical in underpinning replisome movement along the chromosome.

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