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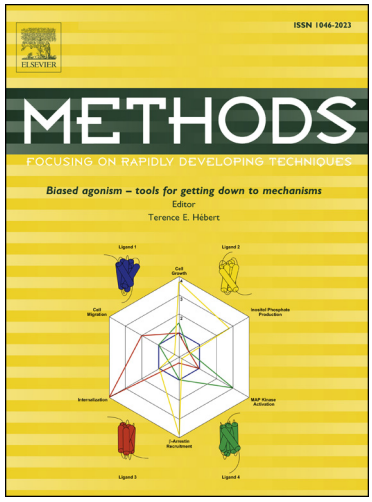

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Use of streptavidin bound to biotinylated DNA structures as model substrates for analysis of nucleoprotein complex disruption by helicases

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Abstract:

Helicases are a subfamily of translocases that couple the directional translocation along a nucleic acid lattice to the separation of nucleic acid duplexes using the energy derived from nucleoside triphosphate hydrolysis. These enzymes perform essential functions in all aspects of nucleic acid metabolism by unwinding and remodelling DNA or RNA in DNA replication, repair, recombination and transcription. Most classical biochemical studies assay the ability of these enzymes to separate naked nucleic acids. However, many different types of proteins form non-covalent interactions with nucleic acids *in vivo* and so the true substrates of helicases are protein-nucleic acid complexes rather than naked DNA and RNA. Studies over the last decade have revealed that bound proteins can have substantial inhibitory effects on the ability of helicases to unwind nucleic acids. Any analysis of helicase mechanisms *in* vitro must therefore consider helicase function within the context of nucleoprotein substrates rather than just DNA or RNA. Here we discuss how to analyse the impact of bound proteins on the ability of helicases to unwind DNA substrates *in vitro*.

- Helicases act on protein-bound nucleic acids *in vivo*
- Simple assays to test displacement of model nucleoprotein blocks from nucleic acids
- Differentiate between protein displacement and DNA unwinding

Keywords:
Motor; DNA unwinding; protein displacement; nucleoprotein complex.

1. Introduction

Helicases are a ubiquitous class of enzymes that disrupt the base pairing between nucleic acid duplexes using energy derived from nucleoside triphosphate (NTP) hydrolysis [1, 2]. The central roles played by helicases in all aspects of nucleic acid metabolism have led to intense efforts to understand the mechanisms by which these enzymes unwind and remodel nucleic acids. We now know that duplex unwinding is achieved by a variety of different mechanisms depending on the class of helicase. For example, unwinding can be achieved by translocation of the helicase along either one or both of the nucleic acid strands [1]. The number of helicase subunits required for unwinding also varies between different types of helicases. Some helicases function as either homohexameric or heterohexameric helicases, especially those associated with the replication machinery [3]. Other helicases are monomeric, although unwinding by many of these monomeric helicases requires multiple monomers to act on the nucleic acid substrate in order to effect unwinding [4]. However, whilst we know much of the detail about how helicases catalyse duplex unwinding, a fact that is often neglected is that nucleic acids are coated with proteins *in vivo*. Whilst some protein-DNA complexes have evolved specifically to block the progression of helicases [5], the majority of nucleoprotein complexes present accidental barriers to helicase movement along their substrates [6-8]. Thus, in addition to the disruption of hydrogen bonding between the nucleic acid base pairs, helicases are also required to break non-covalent bonds between proteins and nucleic acids in order to continue and complete the unwinding of double-stranded nucleic acids. The importance of protein displacement from DNA is illustrated by the dangers posed by nucleoprotein complexes to the completion of genome duplication. For example, replication fork movement in bacteria often stalls due to collisions with protein-DNA complexes, especially those associated with transcription [9-13]. In *E. coli*, movement of the replication machinery is driven by the replicative helicase DnaB. To counter inhibition of fork movement by protein-DNA complexes an accessory replicative helicase, Rep, is also required to allow genome duplication to proceed efficiently [10, 14].

An understanding of how helicases function requires therefore information about how they catalyse both the disruption of base pairing and also disruption of the many non-covalent bonds between the nucleic acid and bound proteins. Site-specific nucleic acid binding proteins can be used *in vitro* to analyse helicase activity on duplex substrates [7, 15-20]. However, the stochastic nature of helicase inhibition by protein-nucleic acid complexes requires higher affinity nucleoprotein complexes in order to facilitate detection of helicase inhibition. The very high affinity interaction between streptavidin and biotin has therefore found favour as a model nucleoprotein barrier when studying helicase activity [21-24]. In addition to the high affinity, the ease with which biotin can be incorporated into synthetic oligonucleotides means that the nucleoprotein barrier can be positioned at any point within a nucleic acid substrate [25]. Moreover, unlike site-specific binding proteins such as *lac* repressor which can only provide a nucleoprotein barrier within the context of double-stranded DNA [7], biotin can be positioned within single-stranded DNA. Thus inhibition of helicase activity by biotin-streptavidin complexes can be studied within the context of both single- and double-stranded nucleic acid. This consideration is important. Disruption of a protein-dsDNA complex requires helicase translocation along the DNA, DNA unwinding and protein displacement whereas disruption of a protein-ssDNA complex requires only translocation and protein displacement. Protein displacement can therefore be studied in the absence of the need to unwind DNA.

Here we describe several approaches for the use of biotin-streptavidin complexes to study helicase activity. We illustrate these approaches by using *E. coli* DnaB and Rep. The use of these two enzymes illustrates how to approach these assays when studying two helicases with opposing polarities but which also interact

physically, an interaction that results in cooperative unwinding of duplex DNA [10].

2. Materials and Methods

2.1. Proteins

Biotinylated Rep (bio-Rep) and DnaB were purified as described previously [10, 26]. Streptavidin was purchased from Sigma-Aldrich and 100 µM stocks were prepared in 25 mM HEPES pH 7.4, 20% glycerol, 10 mM NaCl and stored at -80°C. Proteinase K was purchased from Promega, resuspended in water as a 10 mg ml^{-1} stock and stored at -20°C. Concentrations are those of monomeric Rep and streptavidin and hexameric DnaB.

2.2. Preparation of DNA forks

We use forked DNA substrates to mimic features of the DNA present at a replication fork. The use of forks also allows the same DNA substrate to be used for analysis of helicases with either 3'-5' or 5'-3' polarity. For example, both DnaB, a 5'-3' helicase, and Rep, a 3'-5' helicase, operate at *E. coli* replication forks and forked DNA substrates allows the activities of Rep and DnaB to be compared on an identical substrate. Forked substrates also allow unwinding to be analysed in the simultaneous presence of both helicases, an important consideration if helicases of opposing translocation polarity might function cooperatively.

All oligonucleotides used in this study are indicated in Table 1 and were supplied by Integrated DNA Technologies (IDT DNA). For biotin modifications, internal biotin dT was chosen (IDT DNA Mod code "/iBiodT/").

Oligonucleotide sequences for DNA unwinding assays were based on oligonucleotides 1b-98 and 3L-98 as described in [27] with modifications to contain one or two biotinylated thymines 8 and 9 bp away from the fork junction, as indicated in Table 1.

All oligonucleotides were gel purified. For this, oligonucleotides (1 µg bp^{-1}) were prepared in 40% deionised formamide, 5 mM EDTA, 0.05 mg ml^{-1} xylene cyanol and 0.05 mg ml^{-1} bromophenol blue (final concentrations) as 20 µl reactions and heated to 95°C for 5 min. The reactions were loaded on a 50-55°C warm denaturing 7M urea 12% polyacrylamide gel in a SequiGen apparatus (BioRad) and run at 2100V for 1-3 h depending on their sequence length. After electrophoresis, the gel was wrapped in cling film and oligonucleotides were visualised by UV shadowing at 365 nm. Full length sequences were excised from the gel and eluted in 100 µl of 50 mM Tris-HCl pH 8.0 and 1 mM EDTA (1x TE) at 4°C overnight . The concentration of the eluted, purified oligonucleotides was determined on a NanoDrop 2000 (Thermo Scientific).

To 5' radiolabel the oligonucleotide which contains the free 3' ssDNA arm of the final forked DNA substrate, 25 µl reactions were set up containing 500 ng oligonucleotide, 10 units of T4 Polynucleotide Kinase (PNK; NEB) and 1x PNK buffer (70 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$ and 5 mM DTT). The reactions were incubated at 37°C for 1 h in the presence of 0.4 MBq [γ^{32}P]-ATP (222 TBq $mmol^{-1}$) before heat inactivation of PNK at 65°C for 15 min. Unincorporated [γ^{32}P]-ATP was removed by diluting the reaction to 45 µl with water and passing through a Micro Bio-Spin™ P-6 Gel Column (BioRad) following the manufacturer's instructions, eluting the radiolabelled oligonucleotide in 10 mM Tris pH 7.4. The concentration of radio-labelled ssDNA was assumed to be 90% of the input, due to loss of DNA in the Micro Bio-Spin™ P-6 Gel Columns. In the final 45 µl volume, this gave a concentration of 0.01 µg $µl^{-1}$ in a typical labelling reaction of 500 ng oligonucleotide.

Forked DNA substrates were generated by mixing 20-40 µl radiolabelled oligonucleotide with a threefold molar excess of the complementary oligonucleotide

in 1 x saline sodium citrate (SSC; 0.1 M NaCl and 0.03 M sodium citrate pH 7) buffer in final reaction volumes of 50 µl. Annealing was performed at 95°C for 5 min in a Dri-Block (Techne), followed by slow cooling of the reactions to room temperature (~3-4 hours) by placing the aluminium block on the laboratory bench top. The annealing reactions were separated on a 16x16 cm non-denaturing 10% polyacrylamide 1x TBE gel at 180 V for 90 minutes in a PROTEAN II xi Cell (BioRad). Afterwards, the gel was wrapped in cling film and the corners were marked with luminescent tape. The radiolabelled DNA was visualised by autoradiography, forked DNA was excised from the gel (using the marks from the luminescent tape for orientation) and eluted in 100 µl 1x TE at 4°C overnight.

To calculate the concentration of the DNA fork, radioactive counts per minute (cpm) of 1 µl labelled single-stranded oligonucleotide and 2 µl of the eluted annealed DNA fork were measured on a TriCarb 2900TR Liquid Scintillation Counter (Packard, now PerkinElmer). The cpm μl^{-1} determined for the ssDNA oligonucleotide was divided by its concentration (usually 0.01 µg μl^{-1}), giving cpm μg^{-1} for the single-stranded oligonucleotide. The cpm μl^{-1} of the forked DNA was then divided by the cpm μg^{-1} of the single-stranded oligonucleotide, giving the concentration of the forked DNA in µg μl^{-1}. The concentration of the forked DNA was then divided by the molar mass of the radiolabelled DNA strand to give the molarity of the substrate.

All DNA substrates were diluted to 10 nM in water and stored at 4°C for no more than two weeks to minimise the accumulation of radiation damage.

2.3. Biotin-streptavidin displacement assay

Protein stocks were diluted in 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5 mg ml^{-1} BSA, 10 mM β-mercaptoethanol and 20% (v/v) glycerol. All assays were set up in reaction buffer consisting of 50 mM HEPES pH 8.0, 10 mM DTT, 10 mM magnesium acetate, 2 mM ATP, and 0.2 mg ml^{-1} BSA. Radiolabelled DNA substrates were added to a final concentration of 1 nM. All reactions were carried out at 37°C for 10 minutes in final reaction volumes of 10 µl after the addition of the helicases. Electrophoresis was performed on 16x16 cm 10% polyacrylamide/ 1xTBE gels at 180 V for 90 min. The gels were dried and analysed by phosphorimaging and autoradiography.

The streptavidin concentration required for full saturation was tested by adding streptavidin at the indicated final concentrations in 10 µl reactions containing reaction buffer and 1 nM single-stranded or forked DNA with or without biotin modifications. The reactions were incubated on ice for 5 min to allow the streptavidin to bind to the biotin-modified DNA. The reactions were then shifted to 37°C for 10 min before the addition of 2.5 µl stop buffer (100 mM Tris-HCl pH 7.5, 200 mM EDTA, 10 mg ml^{-1} proteinase K and 0.5% SDS) and analysis by non-denaturing gel electrophoresis to determine the extent of binding of the biotinylated DNA by streptavidin (see above). Note that whilst treatment with proteinase K-containing stop buffer prevented any binding of helicases to the DNA substrates during electrophoresis, DNA-bound streptavidin was resistant to proteinase K treatment. This resistance to proteinase K degradation meant that the presence or absence of bound streptavidin after electrophoresis reflected the ability of the added helicase to disrupt the biotin-streptavidin interaction.

A trap for unbound streptavidin must be added upon addition of a helicase to ensure that any unbound biotinylated DNA generated by helicase catalysis is not simply rebound by streptavidin, giving the false appearance of a lack of streptavidin displacement by a helicase. To assess the concentration of free biotin required to prevent streptavidin rebinding to biotinylated DNA, a 100 mM stock of biotin (Sigma-Aldrich) was prepared in 100 mM Tris-HCl pH 8. A titration of free biotin (10^0, 10^1, 10^2, 10^3 and 10^4 µM final concentration) was performed, either adding it 5 minutes before or after 1 µM streptavidin to the reaction buffer with 1 nM single-stranded or

forked DNA substrates on ice. Once both streptavidin and biotin were added, the reactions were shifted to 37°C for 10 min and terminated with 2.5 µl stop buffer.

For the unwinding and protein displacement assays, the biotinylated DNA was incubated with 1 µM streptavidin for 5 min on ice. Different helicases were then added together with 100 µM free biotin and immediately incubated for 10 min at 37°C prior to being terminated by the addition of 2.5 µl stop buffer. Due to similar migration patterns of the ssDNA-streptavidin and dsDNA products, electrophoresis was performed for 120 min instead of 90 minutes to separate these two species.

The relative amounts of total DNA and product (ssDNA) were determined by measuring band intensities of the phosphorimage scans. Relative ssDNA levels were calculated by dividing the ssDNA measurements by the total DNA intensity in each lane:

$$(1) \quad \frac{ssDNA\ band\ intensity}{total\ DNA\ intensity} = relative\ ssDNA$$

For single-stranded biotinylated DNA substrates the amount of streptavidin-free DNA generated by a helicase was calculated using these relative ssDNA values and correcting for the presence of streptavidin-free DNA in controls lacking helicase (for example, lanes 2, 8 or 14 in Figure 3A):

$$(2) \quad \frac{relative\ SA\ free\ ssDNA\ (reaction\ X) - relative\ SA\ free\ ssDNA\ (no\ helicase\ control)}{1 - relative\ SA\ free\ ssDNA\ (no\ helicase\ control)} = fraction\ SA\ displaced$$

For double-stranded biotinylated DNA substrates DNA unwinding was calculated for reactions without and with streptavidin in a similar manner:

$$(3) \quad \frac{relative\ SA\ free\ ssDNA\ (reaction\ X) - relative\ SA\ free\ ssDNA\ (no\ helicase\ control)}{1 - relative\ SA\ free\ ssDNA\ (no\ helicase\ control)} = fraction\ DNA\ unwound$$

The effect of streptavidin on helicase-catalysed DNA unwinding was given as the fraction of DNA unwound in the presence of streptavidin divided by the fraction of DNA unwound in the absence of streptavidin. Values below 1 indicate inhibition of DNA unwinding by the presence of streptavidin.

Total streptavidin displacement from dsDNA substrates was calculated in a similar manner but used the amount of dsDNA from which streptavidin had been displaced (product ii, Figure 4A) in addition to the amount of streptavidin-free ssDNA:

$$(4) \quad \frac{[relative\ SAfree\ dsDNA\ (X) - relative\ SAfree\ dsDNA\ (no\ helicase)] + [relative\ SA\ free\ ssDNA\ (X) - relative\ SA\ free\ \ ssDNA\ (no\ helicase)]}{1 - [relative\ SA\ free\ dsDNA\ (no\ helicase)\ + relative\ SA\ free\ ssDNA\ (no\ helicase)]} = fraction\ SA\ displaced$$

The calculation of cooperativity in streptavidin displacement by two different helicases is described in the text in section 3.2.3.

3. Results

3.1 Preparing streptavidin-biotinylated DNA structures

It is important to ensure that any biotinylated DNA substrates are saturated with streptavidin. Figure 1A illustrates a gel shift assay using 60mer oligonucleotides having biotin incorporated at the 5' or 3' ends or within the middle of the oligonucleotide, together with a control 60mer lacking biotin. As can be seen, at sub-saturating concentrations of streptavidin multiple slowly migrating streptavidin-DNA complexes can be observed. These are likely to represent single streptavidin tetramers bound by more than one biotinylated oligonucleotide. At higher streptavidin

concentrations these multiple bandshifted species are replaced by a single complex which most likely represents one streptavidin tetramer bound to one oligonucleotide at these higher streptavidin:oligonucleotide ratios.

Having established a saturating concentration of streptavidin, the next step is to determine the amount of free biotin to be added that acts as an effective trap for displaced streptavidin. This ensures that any helicase-catalysed disruption of streptavidin-biotin complexes is not obscured by rebinding of the biotinylated DNA by free streptavidin [23]. To do this, increasing concentrations of free biotin are added to the biotinylated DNA prior to the addition of streptavidin (Figure 1B). This titration establishes the concentration of free biotin that can completely inhibit binding of free streptavidin to biotinylated DNA. A control titration is also performed using the same concentrations of reagents but free biotin is added after addition of streptavidin to the biotinylated DNA (Figure 1B). This second titration ensures that addition of free biotin does not lead to any significant apparent disruption of streptavidin-DNA complexes via trapping of spontaneously dissociated streptavidin. As can be seen, adding excess free biotin after addition of streptavidin has little impact on the apparent stability of the streptavidin-biotinylated DNA complexes (Figure 1B).

The same approach is taken to generate biotinylated double-stranded DNA structures. In our hands the amount of streptavidin needed to saturate biotinylated dsDNA substrates is the same as that needed to saturate biotinylated oligonucleotides (data not shown). However, we have found that streptavidin bound to single biotinylated nucleotides within the double-stranded regions of forked DNA results in streptavidin-biotin complexes that are susceptible to competition upon subsequent addition of free biotin (Figure 2A and B, lanes 8-12). This apparent decrease in stability of the streptavidin-biotinylated DNA complex is abrogated by the presence of a nearby second biotinylated nucleotide on the opposing strand of the duplex region (Figure 2C, compare lanes 8-12 with those in 2A and 2B). We have also observed this difference in one versus two biotinylated nucleotides in a second set of unrelated forked DNA structures (data not shown). We have not explored the reasons for this difference in apparent stability of the streptavidin-biotin interactions but we assume that when two biotin groups are in close proximity within a duplex then a single streptavidin tetramer can interact with both biotin groups simultaneously. Fortunately, having both DNA strands biotinylated has proven useful in dissecting the ability of helicases with opposing polarities to disrupt streptavidin-biotin interactions using the same forked DNA substrate. Having both strands biotinylated does preclude testing the translocation polarity of individual helicases on these substrates but the well-documented polarity of many helicases, both structurally and functionally, often makes such polarity analyses unnecessary. Furthermore, if needed, then polarity effects can be dissected using single-stranded DNA substrates carrying a single biotin [23, 28, 29], also demonstrated below, or using modified forks with only a single ssDNA arm.

3.2 Using streptavidin-biotinylated DNA complexes as model nucleoprotein obstacles

3.2.1 Single-stranded DNA

Unwinding of DNA by DnaB is inhibited substantially by high affinity nucleoprotein complexes such as the *lac* repressor-operator complex whereas Rep is not [7]. This differential ability to unwind protein-bound DNA correlates with the function of Rep *in vivo* in underpinning DnaB-driven replication fork movement along protein bound DNA [10]. To begin to understand how DnaB and Rep function in unwinding protein-bound DNA we have exploited various streptavidin-biotinylated DNA substrates.

The simplest type of streptavidin-biotin helicase block is formed by incorporation of biotin-dT into single oligonucleotides. We use homopolymeric dT_{60} oligonucleotides to avoid intramolecular duplex formation, avoiding the complication of streptavidin displacement potentially requiring duplex DNA unwinding. Use of 60 base oligonucleotides also allows efficient binding of multiple helicase molecules onto the DNA substrates. This is an important consideration given that cooperativity between helicase molecules plays an important role in protein displacement [24]. Thus very short oligonucleotides allowing only a single helicase molecule to catalyse streptavidin-biotin disruption would likely result in little if any displacement [24]. Longer oligonucleotides also facilitate the use of centrally located biotin-dT groups to compare helicases with opposing translocation polarities since relatively extensive regions of single-stranded DNA are present on both sides of the biotin group.

Rep and DnaB have opposing polarities, 3'-5' and 5'-3' respectively [30, 31], and so this must be taken into account when using substrates designed to analyse both helicases. Thus Rep can effectively displace streptavidin from oligonucleotides with a biotin located in the middle of an oligonucleotide or at the 5' end but not when the biotin is positioned at the 3' end of the oligonucleotide (Figure 3A). In contrast, DnaB can displace streptavidin from oligonucleotides containing a biotin at the 3' end or towards the centre of an oligonucleotide but not at the 5' end (Figure 3B). Such comparisons are useful to confirm that the known translocation polarities of the helicases under study correlate with their abilities to displace streptavidin from biotin. As can be seen from Figure 3, such a set of substrates is also informative in comparing the relative abilities of helicases to disrupt streptavidin-biotin interactions. In particular, a 60mer oligonucleotide containing a centrally located biotin-dT can be used to compare relative efficiencies of streptavidin displacement regardless of the translocation polarities of the helicases being compared. Comparing lanes 1-6 in Figure 3A and 3B reveals that Rep is much more effective at displacing streptavidin as compared with DnaB, consistent with previous work [7].

3.2.2 Double-stranded DNA

Duplex DNA substrates containing streptavidin-biotin complexes can also be used to assess the competency of helicases to disrupt the streptavidin-biotin interaction. However, analysis is complicated by the need to consider both displacement of streptavidin and unwinding of the duplex region of the substrate. This is illustrated by the effect of addition of either Rep or DnaB to a forked DNA substrate bearing biotin groups on both strands within the duplex portion of the fork. Both Rep and DnaB unwind forked DNA in the absence of streptavidin, although levels of unwinding are low even when enzyme is in large excess of the DNA substrate due to the low processivities of these two helicases [32-34] (Figure 4A, lanes 3-5 and 9-11; Figure 4B). The addition of streptavidin leads to inhibition of DnaB-catalysed DNA unwinding (Figure 4A, lanes 12-14; Figure 4B and C). In contrast, generation of single-stranded DNA product by Rep is unaffected by the presence of streptavidin (Figure 4A, product iii, compare lanes 3-5 with 6-8; Figure 4B and C).

Closer analysis of the reaction products generated by Rep on streptavidin-bound fork DNA reveals that Rep can displace streptavidin from approximately 50% of all the substrate DNA (bands ii and iii in Figure 4A, lanes 6-8) even though it can unwind only 15% of the substrate (band iii in Figure 4A, lanes 3-5 and 6-8; Figure 4, compare D with B). We conclude from these data that Rep can disrupt streptavidin-biotin interactions within the duplex portion of the fork but may subsequently dissociate prior to completion of unwinding, allowing the partially unwound strands to reassociate and form a fork but without any bound streptavidin (Figure 4E). Streptavidin-free fork DNA probably forms because Rep-catalysed disruption of the interaction between streptavidin and the upper biotinylated strand leaves only the

streptavidin interaction with the lower biotinylated strand which is susceptible to competition by free biotin (see Figure 2A) resulting in streptavidin-free fork DNA (product ii in Figure 4A). Similar experiments on forks having a 25 bp duplex region, rather than the 60 bp duplexes used in Figure 4, result in much greater coupling between fork unwinding and streptavidin displacement (data not shown), as expected if low Rep processivity is the source of the discrepancy between unwinding and streptavidin displacement seen in Figure 4.

These data demonstrate that Rep has an inherently greater ability to displace streptavidin from biotin whilst translocating through double-stranded DNA as compared with DnaB, correlating with the function of Rep as an accessory helicase to aid DnaB-driven movement of the replication fork through protein-bound DNA [10, 11, 14]. Moreover, this differential ability to disrupt a model protein-DNA complex is seen within the context of both single-stranded and double-stranded DNA (Figures 3 and 4). These experiments also highlight the utility of using streptavidin-bound biotinylated DNA as model protein-DNA obstacles for helicases. Such substrates allow comparisons of streptavidin displacement within the context of both single-stranded and double-stranded DNA. Furthermore, a single forked DNA structure harbouring biotin-dT on both duplex strands can be used as a substrate to compare streptavidin displacement by helicases regardless of their translocation polarities.

3.2.3 Analysing helicase cooperativity in streptavidin displacement.

Previous work has established that Rep and DnaB can act cooperatively to unwind forked DNA [10]. This cooperativity is specific to Rep and is not observed using other Superfamily 1 helicases even if they have the same polarity of translocation as Rep [35]. Cooperativity is likely achieved via the physical interaction between Rep and DnaB but the mechanistic basis for this cooperativity is still unclear. Given the function of Rep in promoting DnaB-catalysed movement of the replication machinery through protein-bound DNA, we wished to determine whether Rep and DnaB could act cooperatively in displacing streptavidin from biotin. Use of forked duplex DNA-biotin-streptavidin substrates to test for such cooperativity could potentially display both cooperative DNA unwinding and cooperative disruption of the biotin-streptavidin interaction, complicating interpretation of the data. We attempted therefore to probe for disruption of the biotin-streptavidin interaction by exploiting the ability to position such an interaction within the context of single-stranded DNA. Our previous work had demonstrated that Rep and DnaB could interact physically on a single-stranded dT_n substrate [35]. We reasoned therefore that a biotin positioned in the centre of an oligonucleotide might allow cooperative displacement of bound streptavidin by Rep and DnaB even though they have opposing polarities of translocation (3'-5' and 5'-3' respectively) since both helicases could translocate towards the biotin-streptavidin complex on single-stranded DNA either side of the biotin group. This proved to be the case. Titration of increasing concentrations of DnaB in the presence of 0, 2 or 10 nM Rep demonstrated that there was enhanced displacement of streptavidin when both motors were present as compared with when only one of the two motors was present in the reaction (Figure 5Ai; 5Bi). This cooperativity is most easily visualised by plotting the amount of streptavidin displacement in the presence of both Rep and DnaB divided by the sum of streptavidin displacement by the same concentrations of Rep and DnaB individually (Figure 5Ci). No cooperativity results in an approximate value of 1 on the ordinate regardless of the concentrations of the two helicases (the dashed lines in Figure 5C). Positive and negative cooperativity are indicated by data points above and below this line, respectively. As can be seen in Figure 5Ci, Rep and DnaB display positive cooperativity in removal of streptavidin from a centrally positioned biotin at all tested concentrations of the two helicases.

These data are most easily explained by a model in which Rep and DnaB can

cooperate in streptavidin displacement by translocating towards each other on opposite sides of the biotin-streptavidin complex. This model predicts that relocation of the biotin group to either the 5' or the 3' end of the oligonucleotide would result in absence of cooperativity. Analysis of Rep and DnaB activity on such substrates indicates absence of cooperativity (Figure 5A-C, compare ii and iii with i). Indeed, both 5' and 3' terminal biotin substrates reveal negative cooperativity between Rep and DnaB as indicated by data points below 1 in Figure 5Cii and iii. This negative cooperativity could be explained by inhibition of Rep binding and/or translocation as the single-stranded DNA is bound by increasing amounts of DnaB, although this point has not been explored further.

These data demonstrate the utility of streptavidin-biotin complexes as model nucleoprotein obstacles, in particular the ready ability to position such obstacles within single-stranded DNA substrates. As shown here, this ability is of particular benefit when analysing the complex interplay between two helicases of opposing translocation polarity when displacing proteins from DNA, allowing such displacement to be analysed in the absence of the need to simultaneously unwind double-stranded DNA.

4. Conclusions

Uncovering the mechanisms by which helicases displace proteins from DNA and RNA is challenging from a number of perspectives. A key problem is which nucleoprotein substrate to use as a model. For example, the sheer number of protein-DNA complexes encountered by a replication fork means that even relatively low affinity nucleoprotein complexes could present challenges to genome duplication. However, such lower affinity protein-DNA complexes are poor substrates with which to dissect helicase mechanisms *in vitro* due to their relative instabilities. Conversely, paused and stalled transcription complexes are very high affinity nucleoprotein complexes that present substantial challenges to fork movement *in vivo*. However, generating appropriate helicase substrates using transcription complexes is technically challenging. Moreover, understanding helicase-catalysed protein displacement is facilitated by being able to analyse displacement within the context of both single-stranded and double-stranded DNA, facilitating dissection of protein displacement from DNA unwinding. These factors are addressed by using streptavidin-biotinylated DNA complexes. Biotin can be positioned anywhere within oligonucleotide-based substrates which allows monitoring streptavidin displacement from either single-stranded or double-stranded DNA, as shown here. The high affinity of the streptavidin-biotin interaction also provides a substantial barrier to helicase translocation that allows the use of simple ensemble biochemical approaches to analyse disruption of the streptavidin-biotin interaction *in vitro*. These multiple advantages are highlighted by the relative ease with which cooperative displacement of streptavidin by two helicases with opposing polarities can be detected using the very simple substrate employed in Figure 5. Such model nucleoprotein barriers have the potential to provide key insights into how helicases push proteins off DNA, providing a mechanistic framework with which to understand how helicases deal with nucleoprotein complexes *in vivo*.

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Figure Legends

Figure 1. Assessing saturation binding of biotinylated oligonucleotides by streptavidin and the concentration of free biotin needed to act as a trap to prevent

streptavidin rebinding. (A) 0, 0.01, 0.1, 1 and 10 μM streptavidin was added to oligonucleotides PM326, 327, 328 and 329, all labelled at the 5' end. Binding was monitored by gel electrophoresis. (B) 0.001, 0.01, 0.1, 1 and 10 mM biotin was added to bandshift reactions either before (lanes 3-7) or after (lanes 8-12) addition of 1 μM streptavidin to 5' labelled PM328 and the extent of streptavidin-biotinylated oligonucleotide complex formation assessed by gel electrophoresis. Control reactions in lanes 1 and 2 contained PM328 without and with streptavidin but lacking free biotin.

Figure 2. Determining the relative stabilities of forked DNA structures having biotin-dT on one or both DNA strands. 0.001, 0.01, 0.1, 1 and 10 mM biotin was added to reactions before or after addition of 1 μM streptavidin to radioactively labelled forked DNA substrates. The extent of streptavidin-biotinylated fork complex formation was assessed by gel electrophoresis. Control reactions in lanes 1 and 2 contained forked DNA without and with streptavidin but lacking free biotin. The forks in A-C were formed with 1b-98+CC140B47, CC139B53+3L-98 and CC139B53+CC140B47, respectively.

Figure 3. Comparing the relative abilities of Rep and DnaB in displacing streptavidin from single stranded DNA. (A) Displacement by Rep. The concentrations of Rep were 2, 10 and 50 nM, as indicated. (B) Displacement by DnaB. The concentrations of DnaB hexamer were 2, 10 and 50 nM, as indicated. Streptavidin was present in both (A) and (B) at 1 μM whilst the oligonucleotides used were, from left to right, PM328, PM326 and PM327. The fractions of streptavidin displaced from each of the three biotinylated oligonucleotides by Rep and by DnaB are shown in (C) and (D), respectively.

Figure 4. Displacement of streptavidin within the context of double-stranded DNA. (A) Gel shift analysis of displacement by Rep and DnaB. The concentrations of Rep monomer and DnaB hexamer were 2, 10 and 50 nM, as indicated whilst the concentration of streptavidin, if present, was 1 μM. The fork was formed using CC139B53+CC140B47. The possible products of helicase activity on the streptavidin-bound fork, streptavidin-free forked DNA and single stranded DNA are illustrated on the left. (B) Quantification of the fraction of streptavidin-bound forked DNA unwound to form single stranded DNA. (C) Comparison of the relative levels of forked DNA unwinding catalysed by Rep and by DnaB in the presence and absence of streptavidin. (D) Quantification of the fraction of total streptavidin displaced by Rep. (E) Cartoon illustrating the means by which streptavidin might be displaced from the biotinylated forked DNA without and with complete unwinding of the fork.

Figure 5. Analysis of potential cooperative behaviour in streptavidin displacement from single stranded DNA by Rep and DnaB. (A) Displacement of streptavidin from (i) PM328, (ii) PM326 and (iii) PM327 by Rep and/or DnaB, as indicated. DnaB, when present, was at 2, 10 and 50 nM hexamers whilst Rep monomer concentrations are indicated in nM. Streptavidin was present at 1 μM as indicated. (B) Fractions of streptavidin displaced from (i) PM328, (ii) PM326 and (iii) PM327 at the indicated concentrations of Rep and DnaB. (C) The relative levels of streptavidin displacement when both Rep and DnaB were present in the same reaction versus the sum of displacement in individual reactions containing either Rep or DnaB at the same concentrations. The oligonucleotides are (i) PM328, (ii) PM326 and (iii) PM327. The dashed lines indicate values expected in the absence of cooperativity whilst values above or below this line indicate positive and negative cooperativity, respectively.

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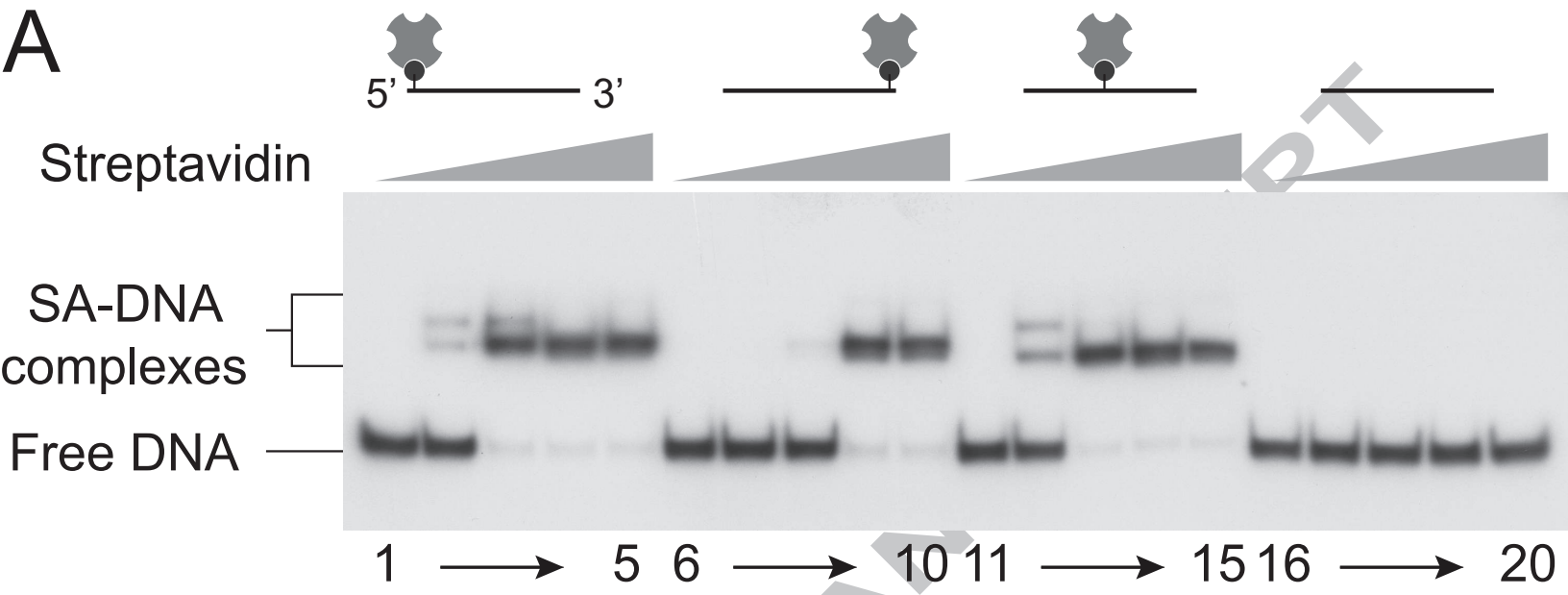


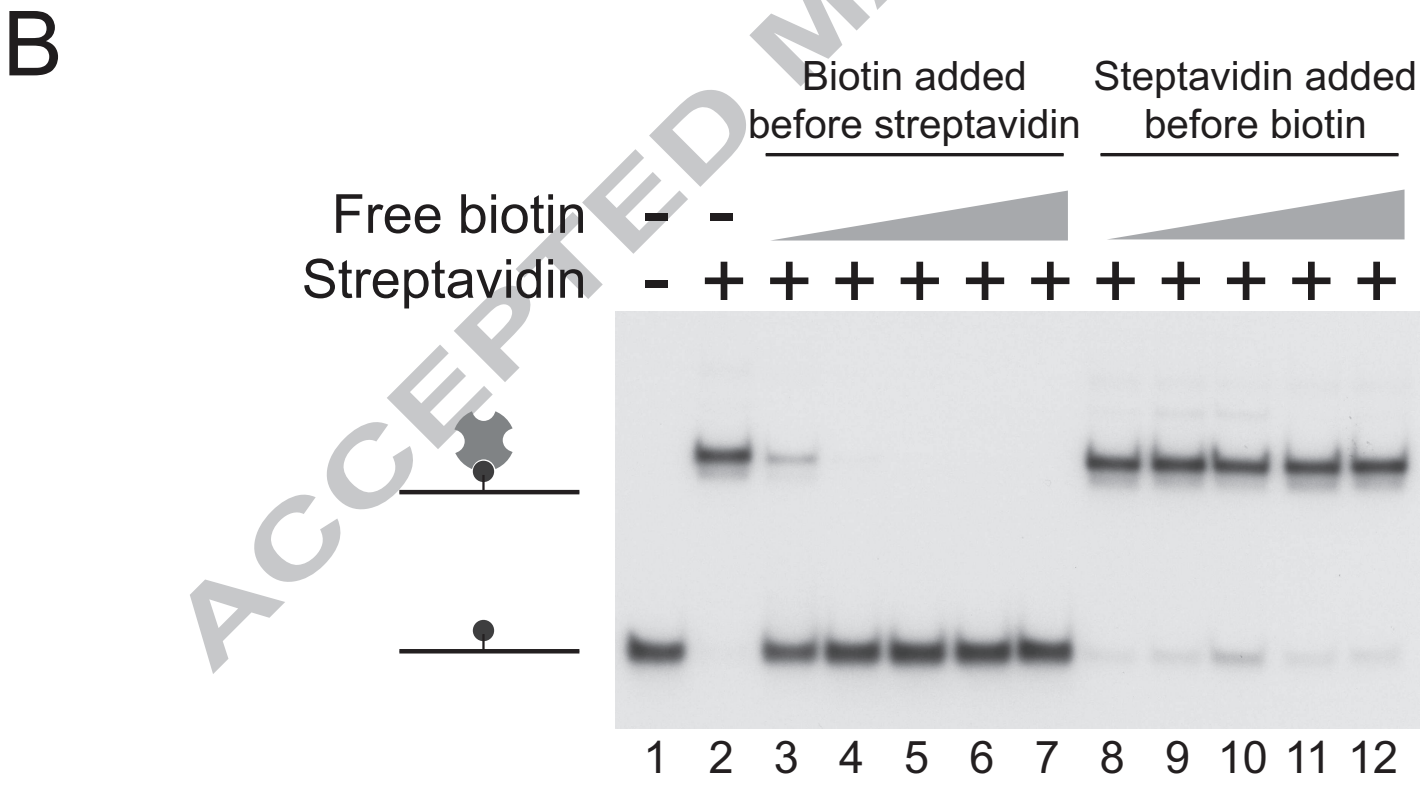


Figure 1

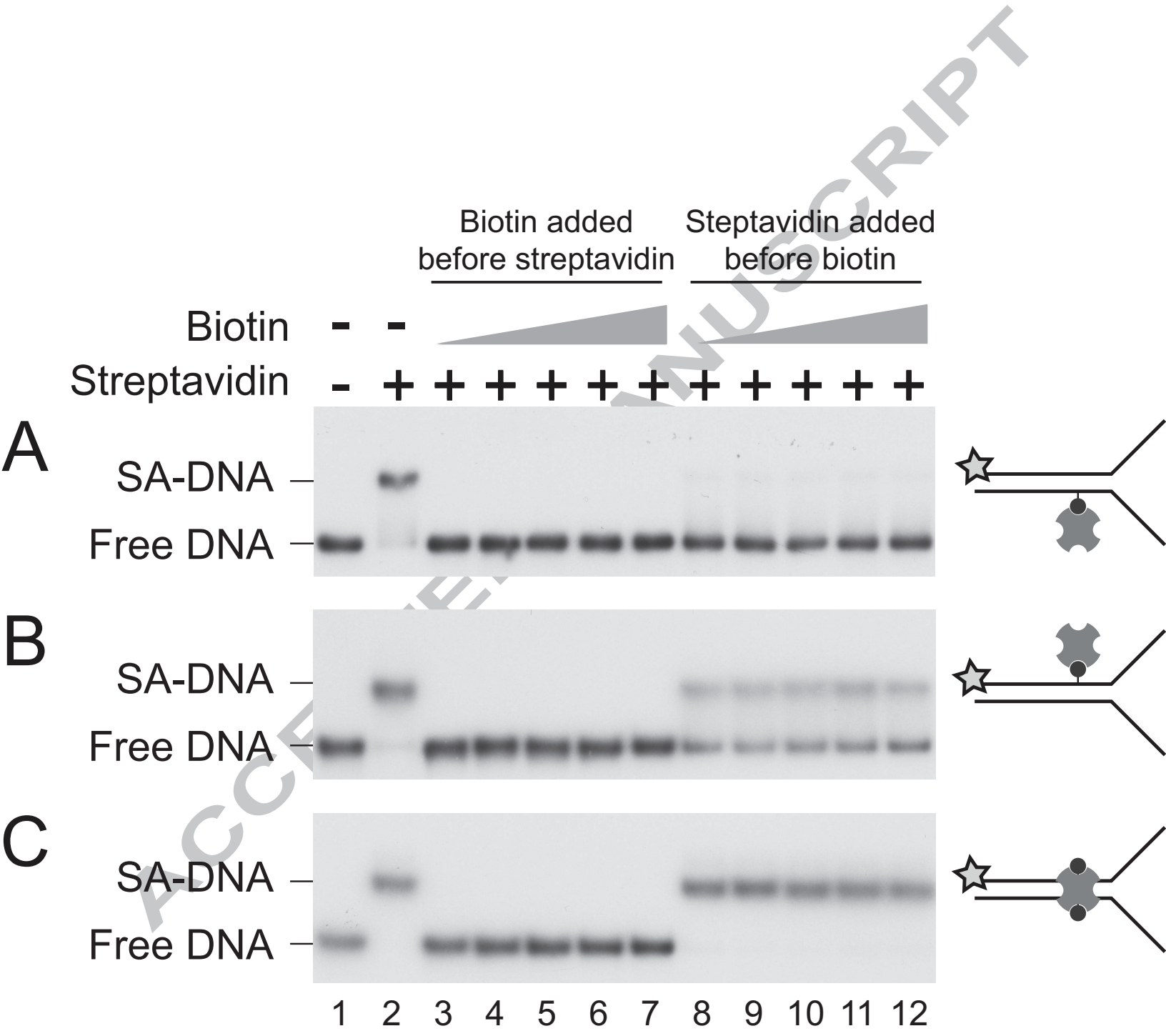
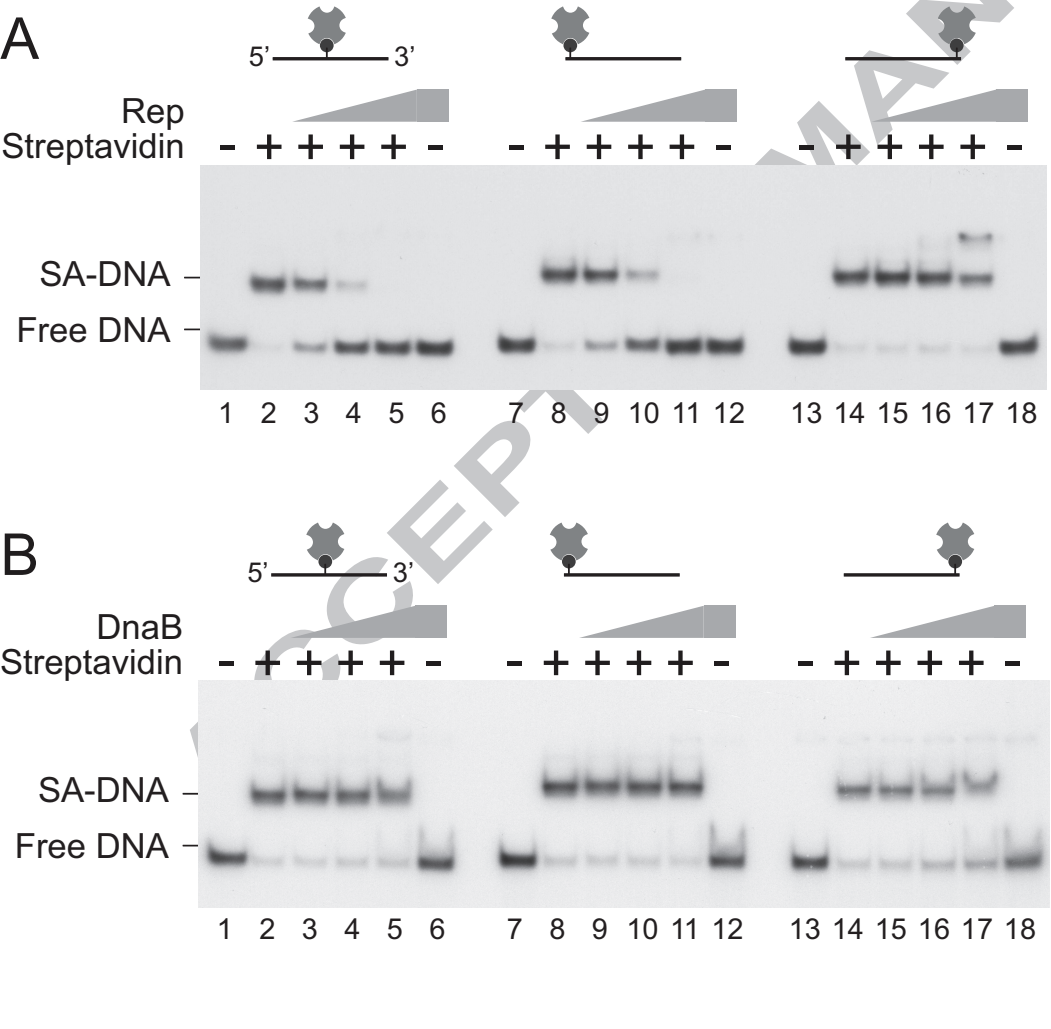


Figure 2



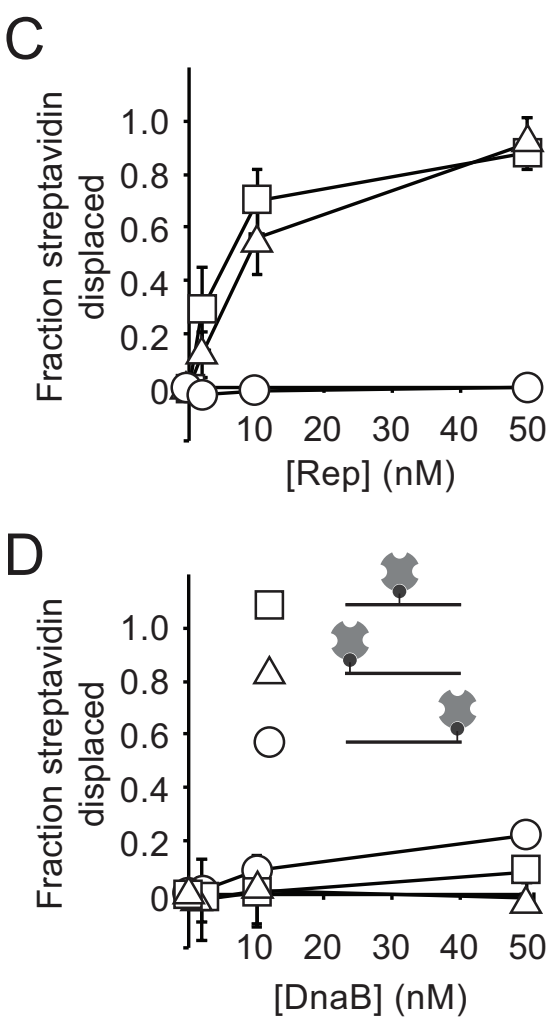


Figure 3

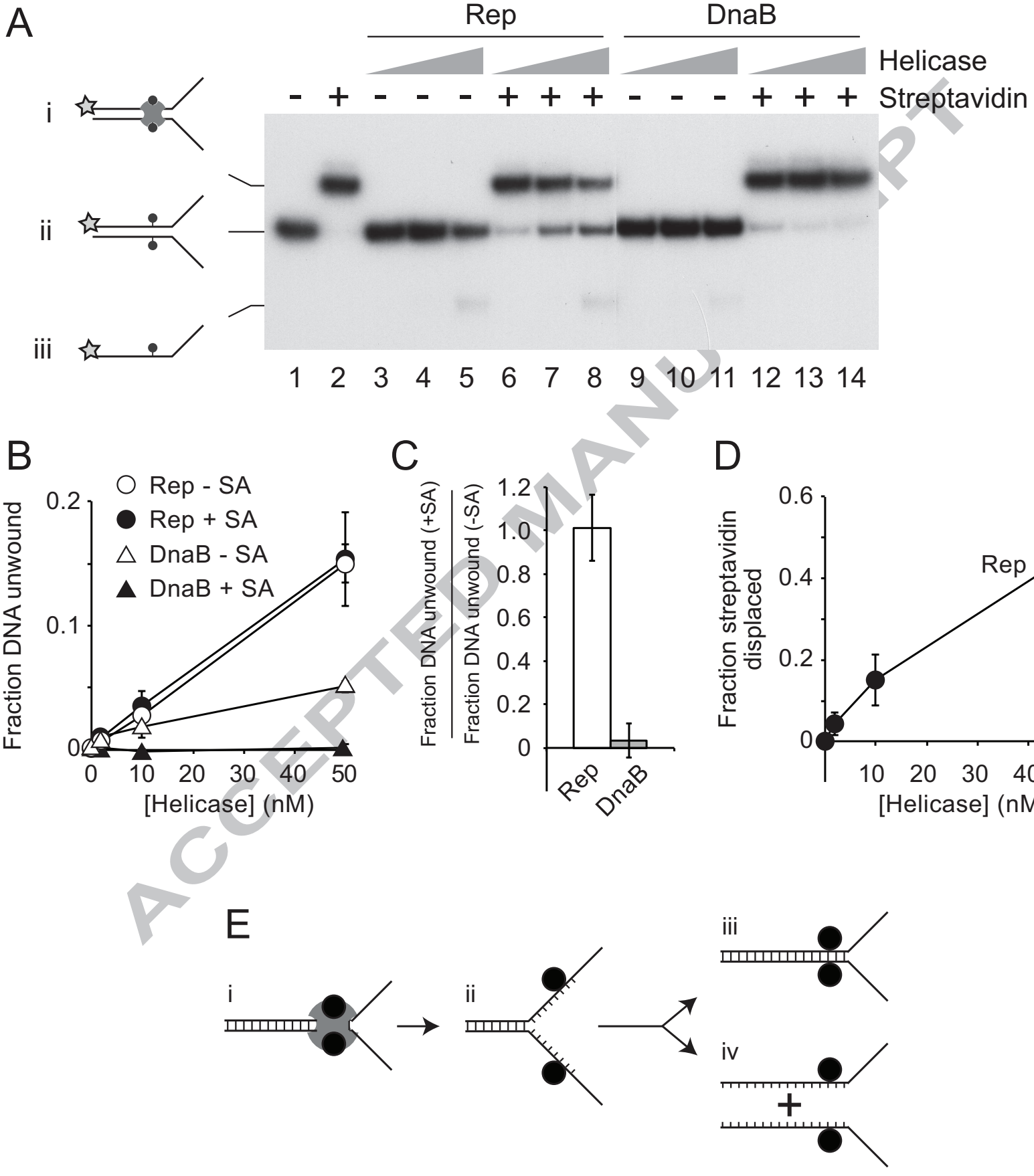


Figure 4

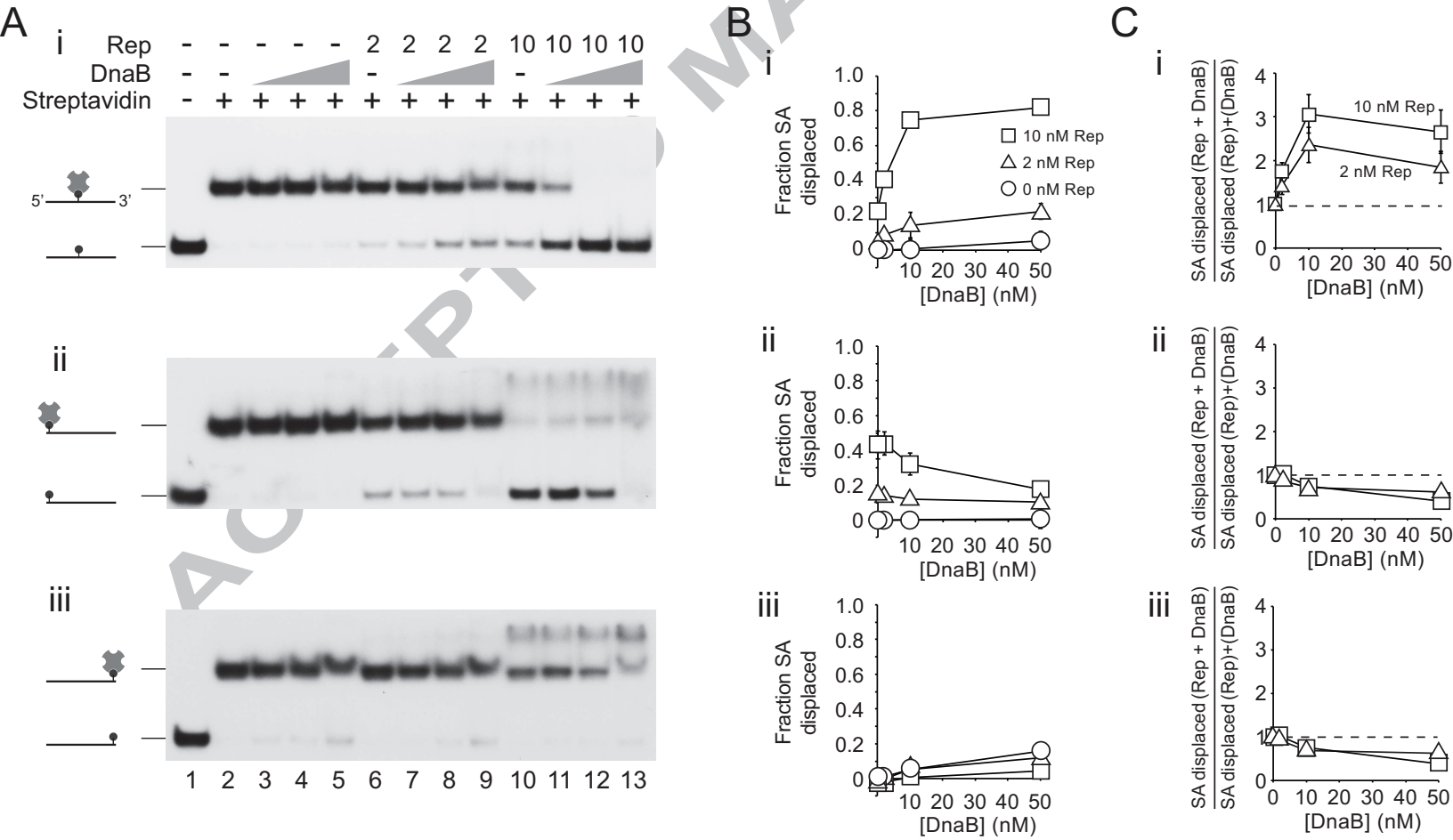


Figure 5

Table 1: List of oligonucleotides

All DNA forks form 60 base pairs dsDNA (underlined sequence) with two free 38 bp long ssDNA arms. "(biodT)" represents a biotinylated thymine.

Name	Sequence (5' – 3')
1b-98	GCAAGCCTTCTACAGGTCGACCGTCCATGGCGACTCGAGACCGCAATACG GATAAGGGCTGAGCACGCCGACGAACATTCACCACGCCAGACCACGTA
3L-98	GACTATCTACGTCCGAGGCTCGCGCCGCAGACTCATTTAGCCCTTATCCG TATTGCGGTCTCGAGTCGCCATGGACGGTCGACCTGTAGAAGGCTTGC
CC139B53	GCAAGCCTTCTACAGGTCGACCGTCCATGGCGACTCGAGACCGCAATACG GA(biodT)AAGGGCTGAGCACGCCGACGAACATTCACCACGCCAGACCA CGTA
CC140B47	GACTATCTACGTCCGAGGCTCGCGCCGCAGACTCATTTAGCCCTTA(bio dT)CCGTATTGCGGTCTCGAGTCGCCATGGACGGTCGACCTGTAGAAGGC TTGC
PM326	T(biodT)TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTT
PM327	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTT(biodT)T
PM328	TTTTTTTTTTTTTTTTTTTTTTTTTTTTT(biodT)TTTTTTTTTTTTT TTTTTTTTTTTTTTTTT
PM329	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTT

Highlights

- Helicases act on protein-bound nucleic acids *in vivo*
- Simple assays to test displacement of model nucleoprotein blocks from nucleic acids
- Differentiate between protein displacement and DNA unwinding