Escherichia coli DbpA Is a 3′ → 5′ RNA Helicase

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ABSTRACT: Previous work has demonstrated that *Escherichia coli* DbpA is a nonprocessive RNA helicase that can disrupt short RNA helices on either the 5′ side or 3′ side of hairpin 92 of 23S rRNA. Here the directionality of the helicase activity of DbpA was determined by using substrates containing a short reporter helix in the presence of a second adjacent helix of varying stability placed either 5′ or 3′ of the reporter helix. When the second helix was on the 5′ side of the reporter helix, it had no effect on the dissociation rate of the reporter helix. However, when the second helix was on the 3′ side of the reporter helix, its dissociation rate determined the dissociation rate of the reporter helix. This defines DbpA as a 3′ → 5′ helicase. Like other helicases, DbpA requires a single-stranded RNA loading site on the 3′ side of the duplex for disruption to be observed. Since the loading site could be on either strand of the helix that was disrupted, hairpin 92 does not influence the directionality of the helicase but only aids in targeting RNA substrates.

Helicases are enzymes that use the energy of ATP hydrolysis to disrupt nucleic acid secondary structures. The classical role of helicases is to unwind DNA or RNA duplexes in advance of the replication machinery (*1*). DNA helicases such as *Escherichia coli* Rep (*2*)and *Bacillus stearothermophilis* PcrA (*3*) and the replicative RNA helicases such as vaccinia virus NPHII (*4*) and HCV NS3 (*5*) are processive enzymes which can unwind many base pairs in a single binding event. Most of the replicative helicases that have been characterized exhibit a 3′ → 5′ directionality as they translocate along the leading strand of the replicative fork (*6*, *7*).

The majority of RNA helicases, termed DExD/H proteins, are not involved in nucleic acid replication but, instead, participate in various facets of RNA metabolism, including translation initiation (*8*), pre-mRNA splicing (*9*), and ribosome assembly (*10*, *11*). These proteins act at specific steps in each pathway and are believed to disrupt inter- and/or intramolecular RNA helices and ribonucleoprotein complexes (*12*, *13*). Since the helices in natural RNA molecules do not generally exceed 20 base pairs, the DExD/H proteins do not need to be very effective helicases. Indeed, experiments with the translation initiation factor eIF4A indicate that it can only efficiently disrupt helices with a free energy of less than −24 kcal/mol, or about 14 base pairs (*14*). *E. coli* DEAD and SrmB, which are involved in 50S ribosomal subunit assembly (*15*, *16*), also can only disrupt short helices (*17*). Similarly, when the stability of the helix joining U1 and U6 RNAs in the spliceosome was slightly increased, it became more difficult for the yeast helicase Prp28p to disrupt the complex (*18*).

The weak helicase activity of DExD/H proteins may also alter the mechanistic properties of the enzymes as compared to the highly processive DNA helicases. For example, if DExD/H proteins use a mechanism similar to the processive helicases and initiate duplex disruption unidirectionally with a step size of 4−6 base pairs (*19*), it may not be necessary for the reaction to proceed beyond this first step. Since most RNA duplexes exist as short helices, melting 4−6 base pairs of a substrate helix may be sufficient to cause the remainder of the helix to spontaneously dissociate. It is also possible that DExD/H proteins do not even translocate along a strand of nucleic acid but, instead, disrupt helices by acting directly upon the end or the middle of the helix (*20*).

Determining the directionality of nonprocessive DExD/H helicase activity is not straightforward. The traditional assay for processive DNA helicases employs relatively long duplex substrates with either 3′ or 5′ single-stranded extensions, termed "tails", that are needed for loading of the enzyme onto the substrate (*21*). The selective disruption of one of these substrates is said to define the directionality of the helicase, or if both constructs are actively disrupted, the helicase is termed bidirectional. However, when this assay is applied to poorly processive helicases, variations in the stabilities and/or sequences of the substrate helices can bias the results. For example, experiments which tested the directionality of the cyanobacterial DExD/H protein CrhC (*22*) used duplex substrates with different lengths and stabilities. The greater stability of the 3′ → 5′ substrate may have unintentionally biased their conclusion that the protein was a 5′ → 3′ helicase. Similar experiments with the yeast splicing protein Prp16 used two-tailed versions of the same duplex and concluded that it was a 3′ → 5′ helicase based on a 3-fold faster dissociation of the substrate with the 3′ tail (*23*). However, it is possible that differences in the fraying equilibria between the terminal G-C and A-U base

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pairs of the two-tailed substrates could be significant enough to lead to different rates of helix disruption (*6*). Finally, while eIF4A is generally considered to be a 3′ → 5′ helicase that needs a single-stranded tail in order to initiate unwinding (*24*, *25*), at least one experiment with eIF4A suggests that no tail at all is necessary for helix disruption (*20*), implying that the protein may be able to act directly on an RNA duplex. Given the multitude of factors that must be taken into account when assaying a nonprocessive RNA helicase by comparing the activity of tailed substrates, it is clear that additional assays are needed to define helicase directionality.

DbpA is an *E. coli* DExD/H protein that demonstrates high affinity and specificity for RNA substrates containing hairpin 92 of 23S rRNA (*26*−*28*). Similar to other DExD/H proteins, DbpA contains all of the conserved sequence motifs that define the family (*29*, *30*) and is likely to fold into the two-domain structure characteristic of the family (*31*). DbpA also contains a unique C-terminal domain which confers the RNA specificity to the protein (*32*). The current model for DbpA interacting with its RNA substrates proposes that the C-terminal domain binds specifically to hairpin 92 and that the catalytic domains can bind to nearby regions of RNA to stimulate ATP hydrolysis and disrupt RNA structures (*28*). Our previous experiments have shown that DbpA can disrupt helices on either side of template strands containing hairpin 92 provided that the RNA duplex is sufficiently short (*33*). Full helicase activity is only achieved when at least 4 single-stranded nucleotides separate the helix from the 5′ end of hairpin 92 or 11 single-stranded nucleotides separate the helix from the 3′ end of hairpin 92. It is unclear whether this interesting asymmetric "spacing requirement" of the helicase activity reflects a bidirectional helicase with different requirements for a single-stranded loading site, a unidirectional helicase with asymmetric binding properties, or a helicase which attacks the duplex directly. Thus, a new approach had to be taken to understand the relationship of the hairpin to the single-stranded loading site and to define the directionality of the helicase activity of DbpA.

MATERIALS AND METHODS

Materials. DbpA was expressed and purified by the protocol of Tsu and Uhlenbeck (*39*). DbpA was concentrated into storage buffer (20 mM MOPS, pH 6.8, 50 mM NaCl, 1 mM DTT, and 50% glycerol) and stored at −20 °C. DbpA concentration was determined by absorbance at 280 nm using a molar extinction coefficient of 27680 M^{-1} cm^{-1} calculated from the amino acid sequence in 6.0 M guanidine hydrochloride.

Plasmid PKK 3535 containing the DNA for domain V of *E. coli* 23S rRNA was amplified using PCR primers to prepare in vitro transcription templates for **1**−**5**. **1**−**3** all consisted of hairpin 92 with a region of domain V appended to the 5′ end of the structure. **1** contains nucleotides 2567−2591, **2** contains nucleotides 2557−2591, and **3** contains nucleotides 2557−2597. **4** consists of rRNA nucleotides 2544−2606 with four mutations: G2599C, G2603C, U2604A, and U2605A. **5** consists of rRNA nucleotides 2544−2596. One of each primer pair contains the T7 promoter in order to generate T7 transcript templates for each of the constructs. After transcription with T7 RNA polymerase (*42*) each template was purified by denaturing 10% polyacrylamide (19:1)/7 M urea gel electrophoresis. All other RNA oligonucleotides were chemically synthesized by Dharmacon Research and 5′-^{32}P-labeled with T4 polynucleotide kinase (from Invitrogen) and [γ-^{32}P]ATP (3000 Ci/mmol).

Bimolecular RNA substrates were formed by annealing each template to the desired short oligonucleotide. Annealing reactions contained 1.5 μM template RNA and 1−3 μM (∼3 μCi) ^{32}P-labeled oligonucleotide, 50 mM HEPES, pH 7.5, and 50 mM KCl in a total volume of 10 μL. After incubation at 95 °C for 60 s and 5 min at 65 °C, $MgCl_2$ was added to a final concentration of 10 mM, and the reaction was cooled at room temperature for 15 min. The annealed RNA was then placed on ice, if to be used immediately, or stored at −20 °C until needed. This protocols yields between 80% and 95% annealed RNA.

Trimolecular RNA substrates were formed using the same annealing protocol as above except that a 4 μM unlabeled third oligonucleotide was added. All trimolecular substrates were run on 22% polyacrylamide (29:1) nondenaturing gels to confirm that efficient formation of the trimolecular complex containing the ^{32}P-labeled oligo was annealed to the template as well as the unlabeled oligo. This protocol yields 75−85% annealed trimolecular RNA substrate. Reactions can be stored at −20 °C with no loss of annealing for up to 2 weeks as long as the reaction is thawed at room temperature and then immediately placed on ice when in use.

Helicase Assays. Protein excess helicase assays were performed at 25 °C under the following conditions: 3 nCi of bi- or trimolecular RNA helicase substrate, 600 nM DbpA, 2 mM ATP·Mg^{2+}, 5 mM $MgCl_2$, 100 μM DTT, 70 μM poly(A), 50 mM HEPES, pH 7.5, 50 mM KCl, and 5% glycerol in a reaction volume of 10 μL. For each time point, the reaction was stopped by adding 9 μL of the reaction directly to 3 μL of quench solution (30 mM EDTA and 0.6% SDS final concentrations). This quench denatures the protein−RNA complex but does not disrupt RNA helices. Aliquots of the quenched reactions (9 μL) were loaded onto a running, nondenaturing 10% polyacrylamide gel (29:1) in 1/3 × TBE. Gels were dried and quantified with a phosphorimager (Molecular Dynamics Storm 820). In all cases control reactions were performed that contained DbpA but lacked ATP. As has been noted previously, these experiments revealed a weak stimulation of RNA annealing with the longer RNA oligomers (>12 nucleotides) (between 3% and 6% over a 30 min time course). All graphs presented represent the average of at least three data sets.

RESULTS

Experiments designed to establish the directionality of DbpA used helicase substrates similar to those studied previously (*33*). The approach was based on that of Jankowsky and Pyle (*4*), who used multipart substrates to determine the step size of the processive RNA helicase NPHII by measuring the lag time for disruption of a short reporter helix in the presence of progressively longer upstream helices created by annealing two oligonucleotides to a common template strand. Since DbpA is not a very efficient helicase and is sequence specific, the experimental design had to be modified in two ways. First, the template strand contained hairpin 92 with either a 3′ or 5′ extension long enough to anneal two oligonucleotides. Second, the rate

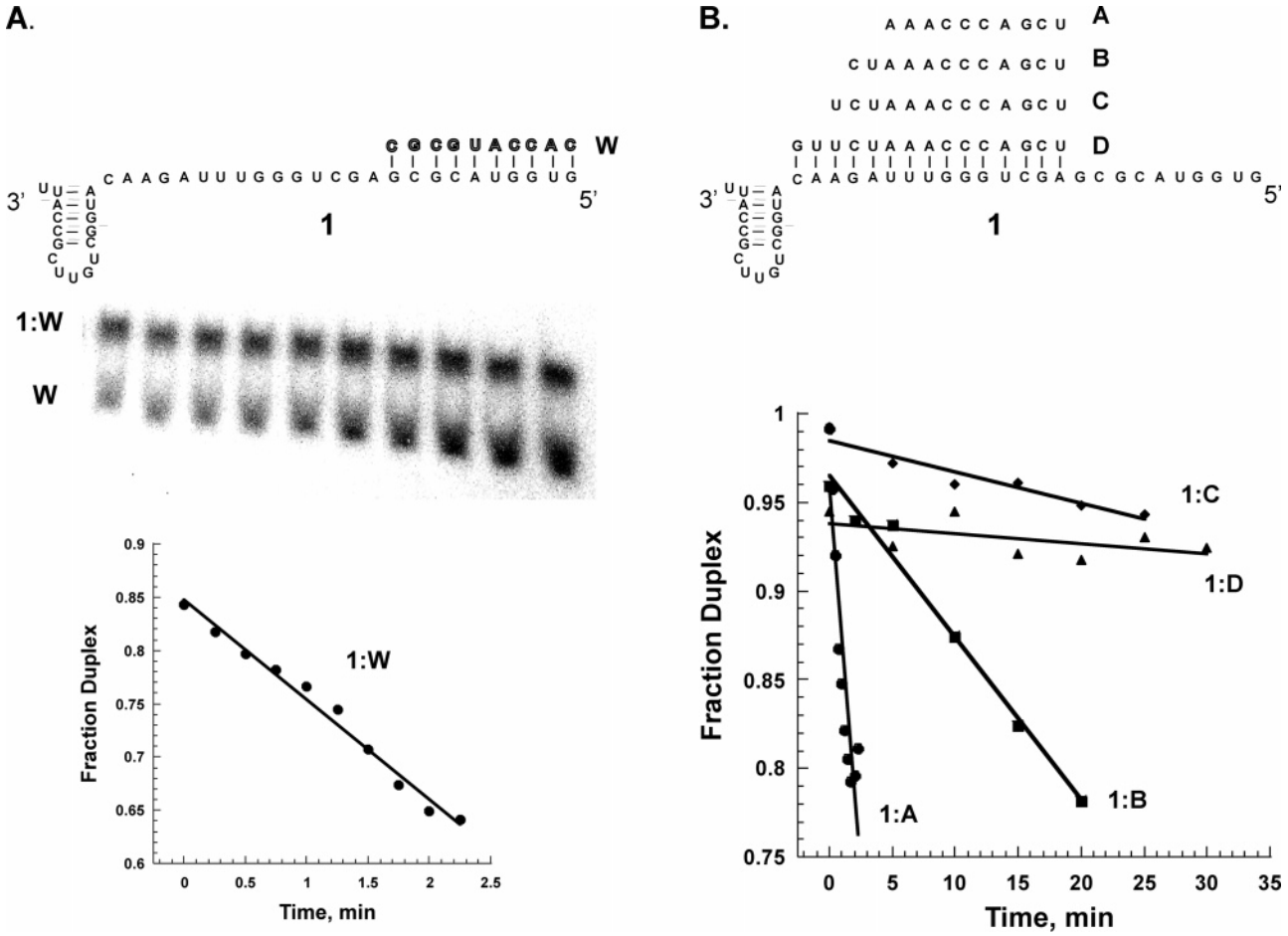

FIGURE 1: Helicase activity of DbpA with bimolecular substrates. (A) A 41-nucleotide RNA consisting of hairpin 92 and a 25-nucleotide region of single-stranded RNA (template **1**) to which oligomer **W** can be annealed to create a helicase substrate for DbpA. DbpA disrupts oligomer **W** from template **1** in a time-dependent fashion. Reactions contain 1 nM bimolecular RNA, 600 nM DbpA, and 2 mM ATP·Mg^{2+}. Disruption of helix **W** can be observed in the gel and is quantitated in the graph below. Average rate of helix **W** disruption = 0.11 ± 0.035 min^{-1}. (B) Template **1** can be annealed to oligomers **A**−**D** to create four different bimolecular helicase substrates. The rate of helix disruption for helices **A**−**D** when annealed to template **1** is shown in the graph: **1**:**A** (●), **1**:**B** (■), **1**:**C** (◆), and **1**:**D** (▲). For average rates, see Table 1.

of disruption of a reporter helix was measured when a second helix of varying stability was placed 3′ or 5′ of the reporter. The first set of experiments used template **1** RNA consisting of hairpin 92 and a 25-nucleotide single-stranded 5′ extension (Figure 1A). When the radiolabeled 10-mer, **W**, is annealed to the extension 15 bases 5′ of hairpin 92, DbpA catalyzes its displacement as detected by nondenaturing acrylamide gel electrophoresis. The observed rate of disruption (0.12 min^{-1}) was similar to that obtained previously using a different substrate with a similar helix length and distance from the hairpin (*33*). The rate of displacement of the reporter oligonucleotide **W** was found to be essentially the same as for **A**, a helix of similar stability, lying five bases 5′ of hairpin 92 (Figure 1B and Table 1). In contrast, three additional oligonucleotides, **B**−**D**, showed substantially slower rates of helix disruption when annealed to the template (Figure 1B). On the basis of previous experiments (*33*) the slower dissociation of helices **B**−**D** is due to two reasons: first, their increased length and stability makes them poor substrates for the weakly processive helicase activity of DbpA, and second, the three oligomers are too close to hairpin 92 for effective disruption to be observed.

The above series of oligonucleotides was then used to create a set of four different trimolecular helicase substrates consisting of template **1** annealed to radiolabeled oligonucleotide **W** and one of the unlabeled oligonucleotides **A**−**D** (Figure 2A). Effective annealing of the second oligonucleotide to the template could be confirmed by its slower migration during nondenaturing gel electrophoresis (Figure 2B). The rate of DbpA-catalyzed displacement of the radiolabeled oligonucleotide **W** from the template was then measured in the presence of saturating levels of ATP·Mg^{2+} and analyzed on nondenaturing gels. As shown in Figure 2C and in Table 1, the rate of displacement of **W** in the trimolecular substrate is very similar to the rates of displacement of **A**−**D** as bimolecular substrates. For example, **W** dissociates about 100-fold slower when helix **C** is present, and this rate is very similar to the rate of dissociation of **C** by itself. This suggests that the oligonucleotides **A**−**D** must be released prior to the dissociation of oligonucleotide **W**.

Table 1: Rates of Helix Disruption by DbpA with Template **1**[a]

oligomer annealed to template **1**	bimolecular substrates (min^{-1})	helix **W** disruption in trimolecular substrates
W	0.12 ± 0.04	
A	0.10 ± 0.05	0.032 ± 0.02
B	0.0034 ± 0.005	0.0042 ± 0.002
C	0.0018 ± 0.002	0.0013 ± 0.0001
D	0.00053 ± 0.0003	0.00065 ± 0.0006

[a] See Materials and Methods for conditions.

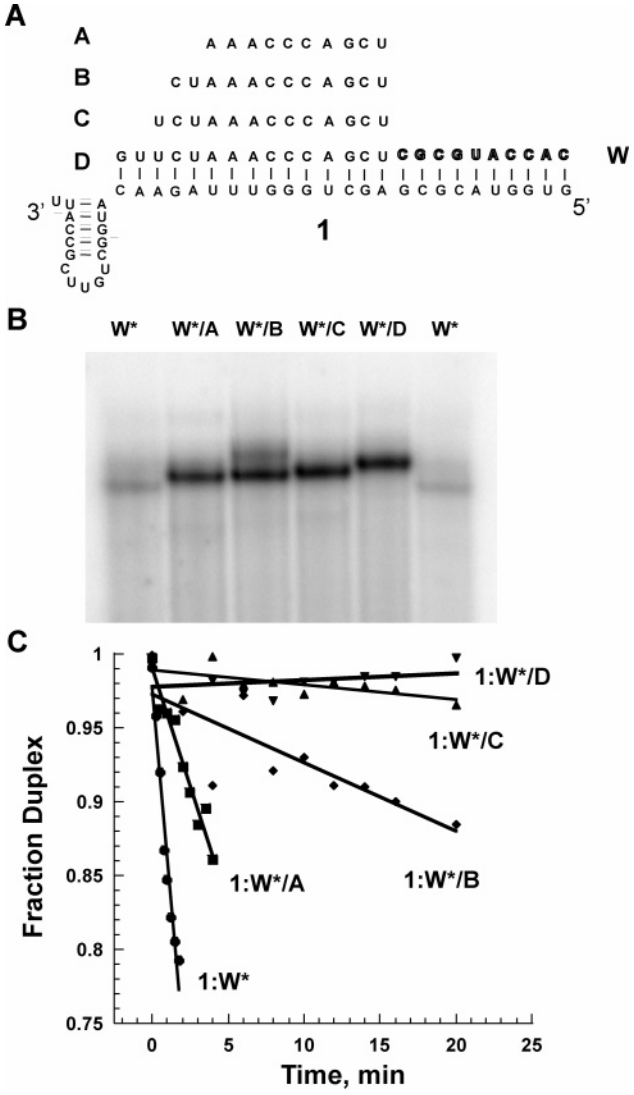

FIGURE 2: Trimolecular helicase substrates. (A) Trimolecular helicase substrates are created by annealing oligomer **W** to template **1** with one of the oligomers **A**−**D**. (B) Nondenaturing gel electrophoresis shows the formation of trimolecular RNA helicase substrates. Oligomer **W** is ^{32}P-labeled in all constructs. Template **1** and oligomer **W** annealed alone (lanes 1 and 6) and template **1**:oligomer **W** with unlabeled oligomers **A**−**D** (lanes 2−5). (C) Disruption of ^{32}P-labeled helix **W** in the context of the trimolecular helicase substrates **1**:**W*** (●), **1**:**W*/A** (■), **1**:**W*/B** (◆), **1**:**W*/C** (▲), and **1**:**W*/D** (▼). For average rates, see Table 2.

This introduction of a rate-limiting step indicates that the helicase activity of DbpA requires a region of single-stranded RNA 5′ to the hairpin and on the 3′ side of the substrate helix in order to effect helix disruption. This region presumably acts as a loading site, and the data therefore suggest that DbpA acts as a 3′ → 5′ helicase on this set of substrates.

Although the above results suggest that DbpA may indeed act as a 3′ → 5′ helicase, they do not exclude the possibility that DbpA could also disrupt helices in the 5′ → 3′ direction since the above substrates lacked a loading site region of single-stranded RNA 5′ of the annealed oligomer **W** that would be needed to initiate unwinding. To test this possibility, a new template, **2**, was created that was identical to template **1** but had an additional 10 nucleotides of single-stranded RNA 5′ of helix **W** (Figure 3A). As summarized in Table 2, the addition of these 10 residues to the template strand did not significantly change the rates of DbpA-catalyzed dissociation of any one of the five oligomers annealed as bimolecular complexes. Furthermore, when the corresponding trimolecular constructs were assayed, the rate of oligomer **W** dissociation again correlated with the dissociation rates of oligomers **A**−**D**. Thus, the presence of the 10 single-stranded nucleotides 5′ to **W** did not result in faster dissociation of **W** in the trimolecular complexes. This experiment suggests that the helicase could not use the introduced 5′ residues as a loading site and proceeds solely in a 3′ → 5′ direction using the loading site on the 3′ side of **W**.

It is possible that the above result reflects the fact that structures of the trimolecular helicase substrates in Figure 3A prevent access of the helicase to the 5′ loading site. For example, the poor activity of the **2**/**D**/**W** substrate may be because its three helices form a coaxially stacked helix that prohibits simultaneous binding of the enzyme to hairpin 92 and the 5′ tail and thereby prevents any 5′ → 3′ helicase activity. In this view, the higher activity of the **2**/**B**/**W** substrate would be because it is sufficiently flexible to allow accessibility to the 5′ tail. To eliminate this possibility, template **3** (Figure 3B) was constructed that contained 6 residues between hairpin 92 and the two annealed oligomers. These extra residues not only provide a flexible linker that should make it more possible for DbpA to access the 5′ leader sequence but also introduce a longer 3′ loading site for oligomers **B**−**D**.

The additional 6 residues did not significantly change the rates of disruption of the bimolecular substrates made with **A**−**D** (Table 3). This indicates that the increased stability of the helices is the major factor preventing unwinding and confirms the poor processivity of DbpA. The rate of dissociation of **W** from template **3** remains essentially the same as with the other templates. Importantly, when the corresponding trimolecular helicase substrates were tested, the rate of helix **W** disruption remained dependent upon the rate of disruption of helices **B**−**D**, indicating that helix **W** disruption was again rate-limited by the other helices. Taken together, the data and experiments presented here demonstrate that DbpA acts as a 3′ → 5′ RNA helicase capable of disrupting short helices 5′ of hairpin 92 as long as the basic 4-nucleotide spacing requirement is met.

Thus far, the directionality of DbpA was only tested with substrates that created helices 5′ of hairpin 92. Since DbpA can disrupt helices 3′ of hairpin 92 as well (*33*), it is of interest to define directionality for these substrates. Since the unique C-terminal domain of DbpA is responsible for the specific interaction of DbpA with hairpin 92, the catalytic domains may be able to interact with any nearby helix as long as a loading site near hairpin 92 is available. If this is the case, then it is possible that DbpA could act as a bidirectional helicase and initiate unwinding at whichever end of the substrate helix is closest to hairpin 92. The observed difference in the spacing requirement for helix unwinding (4 nucleotides for helices 5′ of the hairpin and 11 nucleotides for helices 3′ of the hairpin) could then reflect different loading site requirements for the catalytic domains, which results from geometrical constraints within the protein and the different relative position of the substrate helix to hairpin 92. The other possibility is that DbpA is solely a 3′ → 5′ helicase and the observed 11-nucleotide spacing requirement may be needed to allow the RNA to "wrap

FIGURE 3: Construction of additional trimolecular helicase substrates. (A) Template **2** was constructed to test whether addition of a single-stranded region of RNA 5′ of helix **W** would enable DbpA to disrupt the helix with a 5′ → 3′ direction. (B) Template **3** was constructed to test whether the presence of the four nucleotide spacing requirement would affect the directionality of helicase activity. For disruption rates, see Tables 3 and 4.

Table 2: Rates of Helix Disruption by DbpA with Template **2**

oligomer annealed to template **2**	bimolecular substrates (min^{-1})	helix **W** disruption in trimolecular substrates
W	0.034 ± 0.008	
A	0.11 ± 0.02	0.011 ± 0.007
B	0.0062 ± 0.003	0.0025 ± 0.0009
C	0.0028 ± 0.0005	0.0024 ± 0.001
D	0.00029 ± 0.0003	0.00060 ± 0.0005

Table 3: Rates of Helix Disruption by DbpA with Template **3**

oligomer annealed to template **3**	bimolecular substrates (min^{-1})	helix **W** disruption in trimolecular substrates
W	0.039 ± 0.008	
A	0.13 ± 0.02	0.0057 ± 0.002
B	0.0027 ± 0.004	0.0026 ± 0.0008
C	0.0038 ± 0.003	0.00069 ± 0.00009
D	0.00020 ± 0.0006	$<10^{-5}$

around" and bind to the catalytic domains in the proper orientation for helix disruption.

To test these possibilities, template **4** was constructed that contains hairpin 92 and a 3′ single-stranded RNA extension (Figure 4A). Four different complementary oligonucleotides were annealed to this RNA. All of the helices created with these oligonucleotides are sufficiently far from hairpin 92 to meet the previously established 11-nucleotide spacing requirement. In addition, at least 10 nucleotides of single-stranded RNA were available on the 3′ side of all substrates to serve as a potential loading site. Bimolecular substrates created by annealing either of the shorter oligomers **X** and **Y** provide substrate helices that are efficiently disrupted by DbpA, while substrates made with either of the longer oligomers, **E** and **F**, are too stable for DbpA to disrupt (Figure 4B and Table 4). The trimolecular substrate containing the short radiolabeled oligomer **X** annealed on the 5′ side of the unlabeled oligomer **E** showed rapid disruption of helix **X** by DbpA (Figure 4C). In contrast, the trimolecular helicase substrate created by annealing the radiolabeled short oligomer **Y** on the 3′ side of the unlabeled oligonucleotide **F** resulted in no dissociation of oligomer **Y** by DbpA (Figure 4C). This experiment indicates that DbpA can only act as a 3′ → 5′ helicase on this substrate and uses the 10-nucleotide 3′ tail in order to load and initiate helix unwinding.

To test whether DbpA does indeed use the 10-nucleotide 3′ tail as a loading site, a new template, **5**, was created that deleted the tail, resulting in a blunt-ended substrate helix when **X** was annealed to it (Figure 5A). As shown in Figure 5B, this substrate was inactive with DbpA despite the fact that the same helix is efficiently disrupted when the 3′ extension is present. Interestingly, helicase activity could be restored if the 3′ single-stranded loading site was appended to the displaced short oligonucleotide instead of the template strand. As shown in Figure 5B, oligonucleotide **Z**, which has the same 5′ sequence as **X** but has an additional 10 single-stranded residues on the 3′ end, is displaced from template **5** at a rate similar to the rate of helix **X** displacement from template **4** (Figure 5B). This demonstrates that the 3′ → 5′ helicase activity of DbpA can use a 3′ single-stranded loading site on either strand of the substrate helix.

DISCUSSION

The design of experiments to determine whether the helicase activity of DbpA requires a single-stranded RNA entry site and has a defined directionality was complicated by the fact that active DbpA helicase substrates must contain hairpin 92 of 23S rRNA. While earlier experiments demonstrated that a single-stranded spacing region must be placed between the hairpin and the substrate helix for unwinding to be observed, it was unclear whether these single-stranded regions acted as true helicase loading sites or were simply linkers that allowed the catalytic domains to access the helix. Indeed, the fact that the size of the spacer required for activity depends on which side of hairpin 92 the helix is located suggests that the mechanism of unwinding helices 3′ of the hairpin may be different from that of helices 5′ of the hairpin.

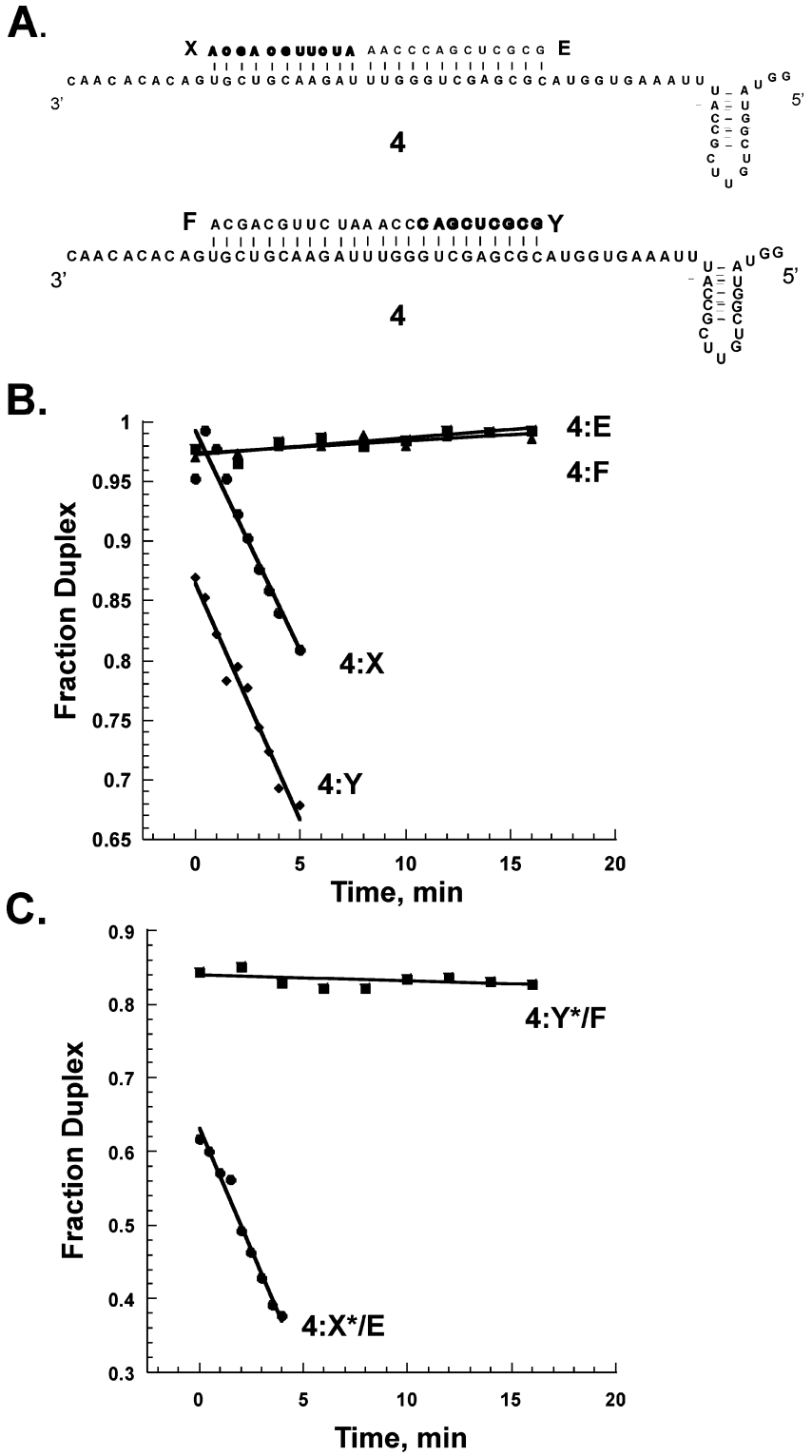

FIGURE 4: Directionality of helicase activity of DbpA with substrate helices 3′ of hairpin 92. (A) Template **4** contains hairpin 92 and can meet all spacing requirements for helix disruption. The combination of oligomers **X** and **E** tests whether DbpA can disrupt helices in a 3′ → 5′ direction. The combination of oligomers **Y** and **F** can be annealed to template **4** to test the ability of DbpA to disrupt helices in a 5′ → 3′ direction. (B) Disruption of helices **X**, **Y**, **E**, and **F** as bimolecular substrates annealed to template **4**: **4**:**E** (■), **4**:**F** (▲), **4**:**X** (●), and **4**:**Y** (◆). (C) Disruption of ^{32}P-labeled oligomers **X** and **Y** in the presence of helices **E** and **F**, respectively: **4**:**X***/**E** (●) and **4**:**Y***/**F** (■). For rates, see Table 4.

As a result it was important with DbpA to supplement the classic "tailed" helicase substrates with a second assay that established the directionality and loading site more directly.

The poor processivity of DbpA was exploited to develop a helicase assay which used trimolecular substrates consisting of a template oligonucleotide containing hairpin 92 annealed to two different oligomers. These experiments have shown that DbpA is exclusively a 3′ → 5′ helicase that requires a single-stranded loading site 3′ of the substrate helix. This means that the essential single-stranded residues 5′ of hairpin 92 do indeed act as a loading site for the catalytic domains. However, the nine single-stranded residues essential for

Table 4: Rates of Disruption of Helices 3′ of Hairpin 92 by DbpA with Template **4**

oligomer annealed to template **4**	bimolecular substrates (min^{-1})	helix disruption in trimolecular substrates
X	0.042 ± 0.009	
Y	0.046 ± 0.003	
E	$<10^{-5}$	
F	$<10^{-5}$	
X*/E		0.031 ± 0.02
Y*/F		$<10^{-5}$

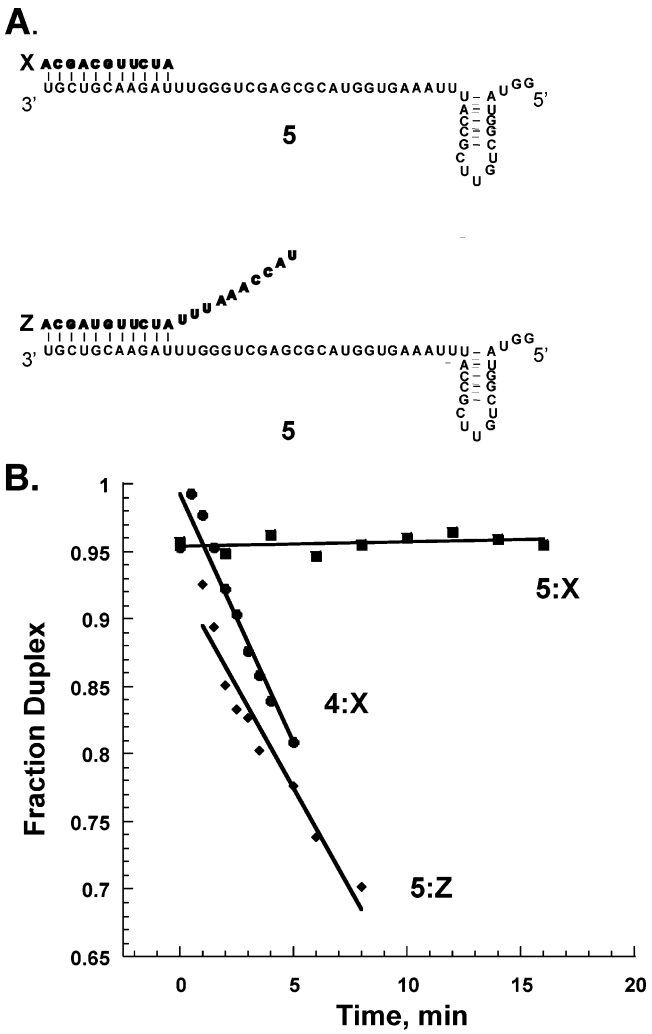

FIGURE 5: DbpA requires a 3′ single-stranded entry site for helicase activity. (A) Template **5** is 10 nucleotides shorter than template **4** and creates a blunt-ended helix at the 3′ end of the bimolecular substrate when annealed to oligomer **X**. Oligomer **Z** has the same sequence as **X** plus an additional 10 residues on the 3′ end which are single stranded and can act as a single-stranded entry site for DbpA. (B) DbpA is unable to disrupt helix **X** in the absence of a 3′ single-stranded region of RNA. Helix **Z**, which contains a 3′ single-stranded entry site, is unwound by DbpA. Rate of helix **Z** disruption = 0.047 ± 0.004 min^{-1}. Key: **5**:**X** (■), **5**:**Z** (◆), and **4**:**X** (●).

unwinding helices 3′ of hairpin 92 do not act as a loading site. Instead, they likely serve as a flexible linker that enables the RNA to wrap around so that the enzyme can interact with the true loading site 3′ of the substrate helix. Indeed, the single-stranded loading site for helices 3′ of hairpin 92 can be in two possible locations: either on the template strand or on the displaced strand. This means that hairpin

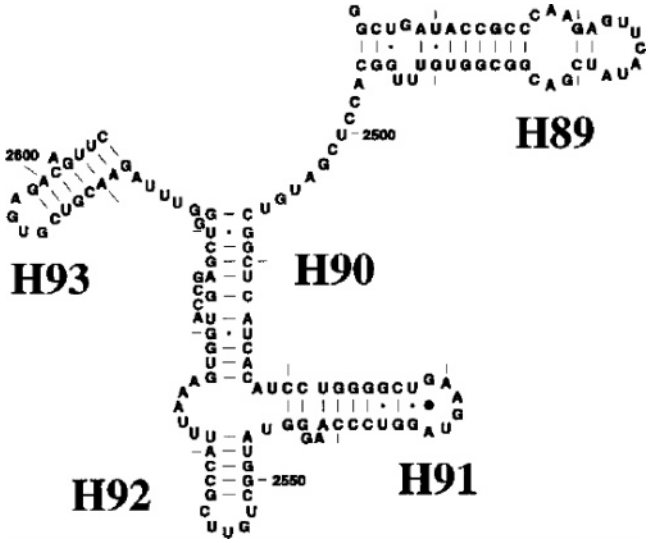

FIGURE 6: The 153-nucleotide fragment of 23S rRNA needed for full DbpA activity.

92 does not influence the directionality of DbpA but, instead, is simply used to recruit the enzyme to its substrate.

It is interesting to note that while DbpA is a poorly processive, sequence-specific RNA helicase, its mechanism appears to resemble that of many of the processive, non-specific, replicative DNA helicases. Thus, like the processive helicases HCV NS3 (*34*), NPHII (*35*), Rep (*36*), PcrA (*3*), and UvrD (*37*), DbpA has the same 3′ → 5′ directionality and requirement for a single-stranded loading site.

The helicase experiments described here are generally consistent with the minimal length requirements for RNA binding to DbpA determined recently (Diges and Uhlenbeck, in press), which defined two different modes for DbpA binding to RNA substrates. In what was termed the direct mode, the protein requires hairpin 92 and at least five adjacent 5′ single-stranded residues similar to the model proposed by Tsu et al. (*28*). A second, inverted, mode of binding is observed for hairpins which have no 5′ single-stranded residues. In this case, at least nine single-stranded residues on the 3′ side of hairpin 92 were needed for binding. It was hypothesized that the longer 3′ single-stranded tail must in some way wrap around to access the single-stranded binding site on the protein. In this paper we show that the helicase activity also can use multiple modes of binding. When the substrate helix is 5′ of hairpin 92, the protein binds to a single-stranded region 5′ to the hairpin in the "direct" mode and processes in a 3′ → 5′ direction. When the substrate helix is 3′ of hairpin 92, the protein binds to a distal single-stranded entry site, analogous to the inverted mode.

The work presented here uses RNA substrates containing only the minimal number of residues needed for the helicase activity of DbpA to be observed. Even though this minimal system is excellent for biochemical and mechanistic studies of the protein, it is important to consider these results in the context of the much larger 23S rRNA substrate. It has been shown previously that a 153-mer consisting of helices 89−93 of 23S rRNA (Figure 6) has a binding and ATPase activity of DbpA similar to that of full-length 23S rRNA (*27*, *39*). The 153-mer binds to DbpA approximately 25-fold tighter than the substrates used in this work and binds cooperatively with ATP, a property not observed with the

smaller substrates (*40*). This suggests that the 153-mer must interact with DbpA in a more complex manner that may more closely mimic its physiological mechanism. Helix unwinding in the context of the 153-mer is presumably also dependent on the presence of appropriate loading sites. The current challenge is to identify the location of the loading sites in this larger RNA. It is striking that in helix 91 a conserved bulge is located four residues 5′ of hairpin 92. This is close to the boundary for helicase entry reported for the minimal substrates (*33*). It is tempting to suggest that this bulge permits loading of the catalytic domains in a manner similar to the minimal substrates **1**−**3** in this work. This would suggest that helix 91 is the physiological target of DbpA. However, the work presented here using model substrates suggests that alternate single-stranded regions may act as loading sites for helicase activity. Indeed, several candidate loading sites have been identified within the 153-mer using footprinting experiments (*41*). These include the bulges in helix 90, the single-stranded regions between helices 89 and 90, and parts of helix 93. This suggests that any one or perhaps all of these nearby regions could be physiological targets of DbpA.

Even though the exact function of DbpA in vivo has yet to be determined, it is believed that DbpA acts at some stage during 50S ribosomal assembly. The unique C-terminal domain of DbpA directs the binding of the protein to hairpin 92, and the interaction of the protein with the folded rRNA structure enhances the affinity of this interaction. Since DbpA disrupts RNA helices exclusively in a 3′ → 5′ direction and a region of single-stranded RNA is required for this activity, it is proposed that DbpA serves to relieve inappropriate conformations during 23S rRNA folding via binding of the catalytic domains to the single-stranded regions within bulges and then initiates unwinding of the inappropriate folded RNA structure. At a later point in the rRNA folding process, DbpA is released from the preribosomal complex and ribosome assembly continues.

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