Trinucleotide repeat expansions are the mutational cause of at least 15 genetic diseases. In vitro, single-stranded triplet repeat DNA forms highly stable hairpins, depending on repeat sequence, and a strong correlation exists between hairpin-forming ability and the risk of expansion in vivo. Hairpins are viewed, therefore, as likely mutagenic precursors to expansions. If a helicase unwinds the hairpin, it would be less likely to expand. Previous work indicated that yeast Srs2 DNA helicase selectively blocks expansions in vivo (Bhattacharyya, S., and Lahue, R. S. (2004) Mol. Cell. Biol. 24, 7324–7330). For example, srs2 mutants, including an ATPase-defective point mutant, exhibit substantially higher expansion rates than wild type controls. In contrast, mutation of another helicase gene, SGS1, had little effect on expansion rates. These findings prompted the idea that Srs2 might selectively unwind triplet repeat hairpins. In this study, DNA helicase assays were performed with purified Srs2, Sgs1, and Escherichia coli UvrD (DNA helicase II). Srs2 shows substantially faster unwinding than Sgs1 or UvrD on partial duplex substrates containing (CTG)(CTG) sequences, provided that Srs2 encounters the triplet repeat DNA immediately on entering the duplex. Srs2 was also faster at unwinding (CAG)(CAG) and (CCG)(CCG)-containing substrates and an intramolecular (CTG)(CTG) hairpin. In contrast, all three enzymes unwind about equally well control substrates with either Watson-Crick base pairs or mismatched substrates with non-CNG repeats. Overall, the selective unwinding activity of Srs2 on triplet repeat hairpin DNA helps explain the genetic evidence that Srs2, not the RecQ homolog Sgs1, is a preferred helicase for preventing expansions.

Expansions of specific DNA triplet repeats are the cause of an increasing number of hereditary neurological disorders in humans (1–3). Unusual genetic mechanisms underlie trinucleotide repeat (TNR)2 instability. Studies from human genetics and from model organisms indicate that the TNR DNA itself plays a major role in its own mutability (1–5). For example, the risk of expansion is closely tied to disease 2, and the risk of expansion increases with the length of the TNR tract and whether the repeat is perfect or imperfect. A central feature of essentially all expansion models (3–6) is that single-stranded TNR sequences fold into aberrant DNA structures, usually hairpins (7), which are crucial intermediates in the mutation process. If a hairpin cannot be prevented from forming or is not removed quickly enough, an expansion will ensue. Thus, hairpins are thought to be direct precursors of expansions.

What is the role of cellular proteins in the expansion process? Proteins that either prevent hairpin formation or accelerate hairpin removal would help reduce expansion rates. One well characterized protein that helps block hairpin formation is the flap endonuclease FEN-1 (8), which cleaves some single-stranded TNR flaps and thereby inhibits expansions (8–13). In addition to its role at TNRs, FEN-1 is also active at many other DNA sequences. Yeast rad27 mutants lacking FEN-1 show pleiotropic effects (14–16), including an unusual mutator phenotype with high rates of duplications and large deletions (17). Thus, although FEN-1 has a potent anti-expansion activity, it functions at many other DNA sequences as well. The mismatch repair factor Msh2 acts in a different way at triplet repeats. Msh2 promotes, not prevents, expansions in transgenic mouse models. When Msh2 is missing in transgenic animals, CAG-CTG expansion frequencies are greatly reduced (18–21). (Curiously, this pro-expansion function of Msh2 is not seen in yeast (22–25) or for the MSH5 function in Escherichia coli (26, 27)). Biochemical experiments show that purified human Msh2-Msh3 heterodimer selectively binds CAG hairpins (28), presumably stabilizing the hairpin and contributing to the expansion process. Thus, the presence of Msh2 is pro-mutagenic under these circumstances, despite the well known anti-mutagenic influence of Msh2 at most DNA sequences.

To help identify cellular factors with the highest possible selectivity for TNRs, we performed an unbiased screen for yeast mutants with elevated rates of TNR expansions. These mutants would presumably identify factors that normally resist expansions. An srs2 mutant was identified (29), and its mutator signature proved to be interesting. srs2 mutants increase expansion rates of CTG, CAG, and CGG repeats up to 40-fold. The expansion phenotype is specific, as srs2 mutants do not alter mutation rates at dinucleotide repeats, at unique sequences, or for the related helicase Sgs1. Disruption of SGS1 did not lead to excess expansions nor did high copy SGS1 suppress the expansion phenotype of an srs2 strain. Sgs1, therefore, does not substitute for Srs2 in blocking expansions in our system. The helicase activity of Srs2 is important because a point mutant lacking ATPase function is also defective in blocking expansions. Purified Srs2 is substantially better than bacterial UvrD helicase at in vitro unwinding of a DNA substrate that mimics a TNR hairpin. We concluded that Srs2 selectively blocks triplet repeat expansions through its helicase activity (29).

Although the selectivity of Srs2 helicase for TNR DNA seems clear, the underlying reasons were not established in the previous study (29). The helicase activity of Srs2 on Watson-Crick partial duplexes has been investigated by several groups (30–34). The enzyme can unwind duplex
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**FIGURE 1. Helicase substrates used in this study.** All partial duplexes were radiola-abeled on the 5′-end of the shorter strand as indicated by the asterisk. Equimolar amounts of the two strands were then mixed and annealed. Base-base mismatches are indicated by larger, bold font. The (CNG)5(CNG)5 substrates tested for unwinding were (CTG)5(CTG)5, (CTG)10(CNG)5, (CTG)5(CNG)10, (CAG)5(CAG)5, and (CAG)10(CAG)5, in all cases, the DNA substrates ran as discrete single bands on non-denaturing polyacrylamide gels, consistent with a well defined structure. As a further test of structure, all (CNG)5(CNG)5, partial duplex substrates and the intramolecular hairpin were subjected to digestion with mung bean nuclease (New England Biolabs) and analysis on high resolution denaturing polyacrylamide gels as described previously (29). The digestion pattern was consistent with the predicted structures.

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**EXPERIMENTAL PROCEDURES**

**Helicase Substrates** — The helicase substrates used for the biochemical assays are listed in Fig. 1. Single-stranded oligonucleotides were purified on denaturing polyacrylamide gels prior to use. The shorter of the two strands was end labeled with 32P using T4 polynucleotide kinase (New England Biolabs) according to the manufacturer’s protocol. The labeled strand was mixed at equimolar concentration with the unlabeled complement, heated at 95 °C for 5 min, and slowly cooled to room temperature to generate the partial double-stranded DNA. To ascertain that the strands had annealed and attained the predicted structure, the proximal (CTG)5(CAG)5 and (CTG)10(CAG)5, partial duplex substrates and the intramolecular hairpin were subjected to digestion with mung bean nuclease (New England Biolabs) and analysis on high resolution denaturing polyacrylamide gels as described previously (29).

**Protein Purification** — Srs2 and Sgs1 were purified to near homogeneity as described (31, 35). Briefly, Srs2 was purified from an overproducing E. coli strain using a six-step procedure including ammonium sulfate precipitation and chromatographic fractionation (31). Expression of His-tagged Sgs1 (amino acids 400–1268 of 1447 full-length protein) was induced by galactose, and the protein was purified using standard nickel chelate chromatography (35). An SDS-polyacrylamide gel showing the purified Srs2 and Sgs1 proteins is provided in Fig. 2. Purified UvrD was a generous gift from Steve Matson, University of North Carolina.

**Helicase Assays** — The helicase assays measure the unwinding of 32P-labeled DNA from a partial duplex DNA molecule. The radiolabeled DNA substrates (0.5 nM) were incubated with 30 nM of the desired helicase for a given period of time, as indicated in the figures, under standard helicase assay conditions. Briefly, Srs2 was incubated with the helicase substrates at 30 °C for varied periods of time in the presence of 25 mM Tris-HCl (pH 7.5), 2.5 mM MgCl2, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, and 2.5 mM ATP. For UvrD, the DNA substrates were incubated with the enzyme at 37 °C, in the presence of 25 mM Tris-Cl (pH 7.5), 3 mM MgCl2, 1 mM dithiothreitol, and 50 μg/ml bovine serum albumin. UvrD was diluted in storage buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 50% glycerol) prior to addition to the reaction mixture. The reaction mixture was preincubated at 37 °C for 5 min, and then unwinding was initiated by the addition of 3 mM ATP. The reactions were terminated with stop buffer (0.3% SDS, 10 mM EDTA, 5% glycerol, 0.1% bromphenol blue). With Sgs1, reactions were performed at 30 °C in the presence of 20 mM Tris-HCl (pH 7.5), 2 mM MgCl2, 2 mM ATP, 2 mM dithiothreitol, and 100 μg/ml bovine serum albumin. Helicase activity was not improved by preincubation of Sgs1 with DNA (5 min at 30 °C, followed by initiation of the reaction by addition of ATP). After incubation at 30 °C, the reactions were terminated with stop buffer (0.5% (w/v) Protease K, 100 mM Tris-HCl (pH 7.5), 200 mM EDTA, 2.5% (w/v) SDS), and the mixtures were incubated at 37 °C for an additional 10 min. To help prevent reannealing of the products of the helicase reactions, a 10-fold molar excess of unlabeled oligonucleotide, which was otherwise identical to the labeled strand, was added at the end of the incubation times. For the intramolecular hairpin substrate, RPA (courtesy of Peter Burgers, Washington University) was added to a final concentration of 150 nM after the reaction, to prevent reannealing of the unwound product. The products of these assays were separated on 12% non-denaturing polyacrylamide gels, followed by phosphorimagining visualization. All assays were performed two to four times with similar results. Graphical representations of helicase assays, aside from those in Fig. 3, are provided as supplemental data.

**RESULTS**

- **Rationale** — Previous genetic and biochemical data indicated that Srs2 helicase selectively blocks expansions of triplet repeats (29). These results suggested that Srs2 helps prevent triplet repeat expansions by unwinding hairpin intermediates and that Srs2 is particularly effective at unwinding triplet repeat DNA. To test this prediction, we performed extensive helicase assays using purified Srs2, Sgs1, and UvrD. Sgs1 was chosen for comparison because Sgs1 and Srs2 provide certain overlapping in vivo functions, such as modulating genetic recombination (36). UvrD was included because it has homology to Srs2 (37) and because UvrD dismantles RecA nucleoprotein filaments (38), similar to the ability of Srs2 to disrupt Rad51 presynaptic filaments (31, 32). In addition, all three enzymes unwind in the 3′ to 5′ direction (30, 35, 39). The helicase activity of Srs2 on Watson-Crick partial duplexes has been investigated by several groups (30–34), but no studies are available on Srs2 unwinding of DNA substrates with non-Watson-Crick duplexes, aside from preliminary data in our earlier study (29). These considerations justify why a comparative study of their unwinding abilities will help ascertain more clearly the biochemical factors driving selectivity of Srs2 for triplet repeat DNA.
Substrates—The helicase substrates used in this study are shown in Fig. 1. These molecules are intended to mimic the triplet repeat hairpin believed to be the crucial expansion intermediate in vivo. Each substrate retains its indicated structure until unwound enzymatically. All duplex substrates share some common features. A 12-nt 3′ single-stranded tail is provided as a helicase loading site. Unwinding by 3′- to 5′-DNA helicases will proceed from the tail into the duplex. Nine complementary base pairs, usually at the distal end of the duplex, add thermodynamic stability and help ensure that the CTG repeats align as predicted. The total duplex length is 24–25 base pairs (including mismatches), aside from the (CTG)_{10}, (CTG)_{15} molecules, which have 39 and 54 base pairs of duplex, respectively. For the intramolecular hairpin, the duplex region is also 24 base pairs. The G+C content is similar for all substrates, aside from the (CCG)_{5}(CCG)_{5} and (ATT)_{5}(ATT)_{5} molecules, which had more and less G+C, respectively. The substrates differ in the composition of most of the duplex region. The control substrate is all Watson-Crick pairs, whereas the (CNG)_{n}(CNG)_{n} partial duplexes and the intramolecular hairpin have single base mismatches every third nucleotide. For additional controls, the (ATT)_{5}(ATT)_{5} molecule represents a trinucleotide repeat sequence with limited hairpin-forming capability that does not expand appreciably in vivo. For the in vitro experiments reported here, duplex formation by the ATT repeats is assisted by the presence of the nine complementary, non-repeating base pairs at the distal end of the duplex. The final test molecule is a non-triplet partial duplex with similar C-G base pairing and T-T mismatches as the (CNG)_{n}(CNG)_{n} substrates but without any repeating three-base nature. There is a hexameric sequence, TGGTCC, repeated twice.

Protein Purification—We purified Srs2 and Sgs1 to near homogeneity (Fig. 2). Purified UvrD was provided as a gift. In all helicase assays described here (with one exception, described later), the three enzymes were tested at equimolar concentrations of 30 nM. The unwinding activity was comparable on a control substrate containing a duplex region of Watson-Crick base pairs (Fig. 3A). All three enzymes unwound the control DNA completely in 10–15 min. Sgs1 was most active on this substrate, followed by UvrD, then Srs2 (Fig. 3C). Thus, the three helicase preparations were deemed active, and the assay conditions, incubation times, and enzyme concentration were suitable for comparison on triplet repeat-containing substrates.

Srs2 Unwinds TNR Substrates Faster than UvrD and Sgs1—We examined the (CTG)_{5}(CTG)_{5} substrate, where part of the duplex is composed of (CTG)_{5} sequences on both strands. We chose the five-repeat length to verify earlier biochemical findings (29) that Srs2 can unwind (CTG)_{5}(CTG)_{5} duplex repeat structures. In addition, a hairpin with about five repeats on each strand mimics what a single-stranded (CTG)_{13} repeat tract might adopt in vivo, and Srs2 was found to be highly active at inhibiting expansions of (CTG)_{13}. The products of the unwinding assays are shown in Fig. 3B. Srs2 unwound 90% of the substrate in 2 min, with complete unwinding achieved in 5 min. In contrast, UvrD and Sgs1 still show 20–40% substrate remaining at 5 min, and traces of substrate remain at 10 min. In contrast to the earlier finding (Fig. 3A) that Srs2 was somewhat slower on control DNA, the results in Fig. 3B support the idea that Srs2 at equimolar concentration is more active than UvrD or Sgs1 at unwinding CTG repeat-containing DNA duplexes. Quantitative assessment of unwinding showed that Srs2 unwinding of the control DNA substrate occurs at about 80–90% the rate of the other two enzymes (Fig. 3C), but Srs2 is ~3-fold faster on the (CTG)_{5}(CTG)_{5} partial duplex (Fig. 3D). When assayed over a range of protein concentrations (Fig. 4), Srs2 consistently unwound the (CTG)_{5}(CTG)_{5} partial duplex 3-fold faster than UvrD and Sgs1. Thus, Srs2 preferentially unwinds this TNR-containing duplex at moderate enzyme concentrations (up to 30 nM). When the enzyme concentration was increased to a very high level (200 nM), all three helicases completely unwound the (CTG)_{5}(CTG)_{5} partial duplex within 5 min (not shown). This indicates that high enzyme levels are necessary to overcome the slow unwinding of the triplet repeat duplex by UvrD or Sgs1.

To determine whether the enhanced unwinding of CTG repeat substrates by Srs2 was true with longer repeat tracts, we tested substrates containing (CTG)_{10}(CTG)_{10} and (CTG)_{15}(CTG)_{15} in the duplex.
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region. The rationale for this experiment is based on genetics (29), which showed srs2 mutants are raised for expansion rates from starting tracts of (CTG)10 but the magnitude of the phenotype is not as large as for (CTG)15 alleles. Thus, we might expect that Srs2 unwinding would be slower when more CTG repeats are included in the helicase substrates. Srs2 was capable of completely unwinding the (CTG)10×(CTG)10 and (CTG)15×(CTG)15 substrates in 10 and 15 min, respectively (Fig. 5, A and B). Thus, more time is required for Srs2 unwinding as the CTG tract is increased from 5 to 10 repeats. This is partially a consequence of the longer duplex regions (24, 39, and 54 base pairs). The duplex length does not account for all the increased time requirements, as the enzyme has been shown to unwind up to 145 base pairs of Watson-Crick DNA even at lower enzyme concentrations (30) than used in Fig. 5. Nonetheless, Srs2 was still significantly faster than UvrD or Sgs1 at unwinding (CTG)10 DNA substrate faster than either UvrD or Sgs1. For Srs2, unwound products were completed (90%) at 15 min of reaction. Product formation at 15 min was the same for (CTG)15 DNA substrate as for (CTG)13 alleles. Thus, we might expect that Srs2 unwinding was completed at 10 min. The unwinding rates for UvrD and Sgs1 had unwound only about one-half the DNA at that time. Similarly, Srs2 is more active than the other two helicases on (CCG)5×(CCG)5 DNA (Fig. 7B), although none of the enzymes caused complete unwinding by 15 min (45% unwinding by Srs2, 10% by UvrD, and 20% by Sgs1). A control experiment (not shown) with RPA present did not improve unwinding of the (CCG)5×(CCG)5 substrate. This control reduces the likelihood that the CCG repeat-containing strands were actually unwound but then reannealed behind the helicase. Together, these observations further support our finding that Srs2 is the most active of the three helicases on TNR substrates. All three enzymes show an apparent sequence specificity of CTG > CAG > CCG.

Possible Mechanism of Action of Srs2—To help ascertained biochemical properties that drive the selectivity of Srs2 for TNR sequence and/or structure, two other substrates were tested. First, we asked whether a triplet repeat per se is important for the selective unwinding activity of Srs2. A non-triplet substrate was created (Fig. 1) with duplex length, C-G base pairing, and T-T mismatches at every third position, similar to the (CTG)5×(CTG)5 substrate on which Srs2 was highly active. However, the new substrate was not a simple CTG repeat; instead, there is a hexameric sequence, TGGTCC, repeated twice. This choice of sequence is largely a result of the constraints listed above. Interestingly, we found that the activity of Srs2 was somewhat compromised. Srs2 unwinding of the non-triplet repeat substrate (Fig. 8A) was observed at 5 min of incubation, and the substrate was only 60% unwound after 10 min of reaction. Both UvrD and Sgs1 were faster than Srs2, catalyzing 95% unwinding of the substrate within 10 min of reaction. This pattern more closely resembles what was seen with the control Watson-Crick duplex in Fig. 3A rather than the expedited unwinding by Srs2 for CNG-containing duplexes. Next, we looked at a substrate containing (ATT)5 (Fig. 8B). The (ATT)5×(ATT)5 molecule represents a trinucleotide repeat sequence with limited hairpin-forming capability (7, 40) that does not expand appreciably in vivo. We found that with Srs2 unwinding was completed at 10 min. The unwinding rates for UvrD and Sgs1 were slightly faster than Srs2, as evident from the 5-min time point (68% product formation by Srs2, 90% by UvrD, and 85% by Sgs1). Taken together, the results from Fig. 8 suggest that the selective unwinding of CNG repeat substrates by Srs2 is not recapitulated by mimicking the structure of the duplex with non-CNG repeat DNA.

FIGURE 4. Unwinding of (CTG)5×(CTG)5 DNA substrates as a function of enzyme concentration. Srs2, UvrD, and Sgs1 at the indicated concentrations were incubated with the (CTG)5×(CTG)5 DNA substrate (0.5 mm) for 0–10 min. Quantitation of unwinding was performed as described in the legend to Fig. 3, and the percent unwinding per minute of reaction was calculated for the linear response range. Error bars are the range of values observed for two-three repetitions of each experiment. Filled circles, Srs2; unfilled diamonds, UvrD; unfilled circles, Sgs1.

FIGURE 5. Effect of longer (CTG)×(CTG) tracts on helicase activity. Helicase assays were performed as described earlier. A (CTG)×(CTG)× containing DNA 8, the substrate contained (CTG)5×(CTG)5 within the duplex. For both substrates, the thicker lines represent the position and approximate length of the CTG repeats within each strand.

Effect of Srs2 on an Intramolecular Hairpin Substrate—Next we examined an intramolecular hairpin substrate, which is identical to the (CTG)5×(CTG)5 substrate but contains a pentanucleotide A loop at the end (Fig. 6). This structure is intended to more closely resemble an in vivo hairpin structure; however, its intramolecular nature might favor reannealing behind the helicase. To help prevent such an occurrence, RPA was added to the reaction mixture to trap the unwound DNA in the single-stranded form. The results (Fig. 6) show that Srs2 unwound the substrate faster than either UvrD or Sgs1. For Srs2, unwound products became visible after 5 min of incubation, and unwinding was almost complete (90%) at 15 min of reaction. Product formation at 15 min was only 30% for UvrD and 55% for Sgs1. Overall, we saw more rapid unwinding by Srs2 than the other two enzymes. The relatively slow unwinding of this substrate by all three proteins indicates that the presence of the loop may reduce helicase activity.

Effect of Srs2 Helicase on Different TNR Sequences—Genetic analysis (29) indicates Srs2 is capable of blocking expansions of several CNG repeat tracts. If this is due to hairpin unwinding, Srs2 helicase should be active on other (CNG)×(CNG) substrates besides the CTG repeat molecules tested so far. The results of unwinding assays with a (CAG)5×(CAG)5 partial duplex target are shown in Fig. 7A. Srs2 nearly completed unwinding (95%) by 15 min, whereas UvrD and Sgs1 had unwound only about one-half the DNA at that time. Similarly, Srs2 is more active than the other two helicases on (CCG)5×(CCG)5 DNA (Fig. 7B), although none of the enzymes caused complete unwinding by 15 min (45% unwinding by Srs2, 10% by UvrD, and 20% by Sgs1). A control experiment (not shown) with RPA present did not improve unwinding of the (CCG)5×(CCG)5 substrate. This control reduces the likelihood that the CCG repeat-containing strands were actually unwound but then reannealed behind the helicase. Together, these observations further support our finding that Srs2 is the most active of the three helicases on TNR substrates. All three enzymes show an apparent sequence specificity of CTG > CAG > CCG.

Possible Mechanism of Action of Srs2—To help ascertained biochemical properties that drive the selectivity of Srs2 for TNR sequence and/or structure, two other substrates were tested. First, we asked whether a triplet repeat per se is important for the selective unwinding activity of Srs2. A non-triplet substrate was created (Fig. 1) with duplex length, C-G base pairing, and T-T mismatches at every third position, similar to the (CTG)5×(CTG)5 substrate on which Srs2 was highly active. However, the new substrate was not a simple CTG repeat; instead, there is a hexameric sequence, TGGTCC, repeated twice. This choice of sequence is largely a result of the constraints listed above. Interestingly, we found that the activity of Srs2 was somewhat compromised. Srs2 unwinding of the non-triplet repeat substrate (Fig. 8A) was observed at 5 min of incubation, and the substrate was only 60% unwound after 10 min of reaction. Both UvrD and Sgs1 were faster than Srs2, catalyzing 95% unwinding of the substrate within 10 min of reaction. This pattern more closely resembles what was seen with the control Watson-Crick duplex in Fig. 3A rather than the expedited unwinding by Srs2 for CNG-containing duplexes. Next, we looked at a substrate containing (ATT)5 (Fig. 8B). The (ATT)5×(ATT)5 molecule represents a trinucleotide repeat sequence with limited hairpin-forming capability (7, 40) that does not expand appreciably in vivo. We found that with Srs2 unwinding was completed at 10 min. The unwinding rates for UvrD and Sgs1 were slightly faster than Srs2, as evident from the 5-min time point (68% product formation by Srs2, 90% by UvrD, and 85% by Sgs1). Taken together, the results from Fig. 8 suggest that the selective unwinding of CNG repeat substrates by Srs2 is not recapitulated by mimicking the structure of the duplex with non-CNG repeat DNA.
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Position Effect of the TNR Sequence on Srs2 Activity—The unusual preference for CNG repeat-containing substrates by Srs2 compelled us to study whether the position of the CNG sequence within the duplex plays a role in determining helicase specificity in our in vitro assay. Based in part on the control experiments in Fig. 8, we postulated that Srs2 selectively unwinds duplexes where the CNG repeat sequence occupies the proximal end of the duplex. If so, changing the position of the CNG repeat sequence within the duplex to the middle or distal end might negate the advantage of Srs2 as a helicase. This prediction was borne out by experimentation. All three enzymes were about equally active when the (CTG)₅ sequence was positioned in the middle (Fig. 9A). Unwinding was 59–72% by 10 min and completed at 15 min. In other words, the helicase activity of Srs2 was slowed compared with when the (CTG)₅ sequence is proximal (Fig. 3, B and D, with 90% unwinding by 2 min). This slowing was even more dramatic when the (CTG)₅ sequence is placed at the distal end of the duplex region (Fig. 9B). Only 45% of the DNA molecules were unwound by Srs2 at 15 min of reaction. In contrast, UvrD and Sgs1 were just as active on this substrate (Fig. 9B; 70–75% unwound at 10 min, 95–99% product formed at 15 min) as when the (CTG)₃(CGT)₅ tract is centrally positioned (Fig. 9A). These data suggest that something about the (CTG)₅(CGT)₅ duplex impedes most helicases when it is encountered proximally but Srs2 can overcome this inhibition. When (CTG)₅ is encountered later in the duplex, Srs2 loses its advantage as a helicase.

DISCUSSION

This study demonstrates that purified Srs2 is substantially more active (~3-fold) at unwinding CNG repeat substrates compared with two other helicases. This feature was observed both for partial duplex substrates and for an intramolecular hairpin. The selectivity of Srs2 helicase activity in vitro correlates with its signature mutator phenotype in vivo, where only CNG repeat expansion rates are elevated in srs2 mutants (29). In contrast, Sgs1 helicase and UvrD were significantly less active at unwinding CNG repeat substrates in vitro, and sgs1 mutants have virtually no impact on expansions in our system (29). The differences in helicase activity of Srs2, Sgs1, and UvrD could not be attributed to trivial reasons because all three enzymes were similarly active on non-CNG repeat substrates. These facts support the idea that CNG repeat structures are unusually difficult to unwind but that Srs2 can do so with higher efficiency. This biochemical property of Srs2 no doubt contributes to, but does not explain fully, its characteristic effectiveness at blocking triplet repeat expansions in yeast. Nonetheless, this novel feature of Srs2 helicase on triplet repeat sequences provides a new par-
adigm for cellular mechanisms that help prevent biomedically relevant mutations.

Unwinding of unusual DNA structures by specific helicases is well established. For example, RecQ family members have been shown to unwind a wide range of substrates (41–43). One example that is especially relevant for our study is the ability of WRN to unwind tetrahelical structures associated with (CGG)$_n$ repeats (44). In addition, the ability to unwind these structures has been associated in some cases with increased processing by associated enzymes, such as nuclelease digestion (45, 46) or DNA polymerization (47). Thus, there is ample precedence for the idea that a subset of helicases is especially active on unusual DNA structures and that this action promotes normal metabolism of the DNA. The novelty of our observation is that Srs2 is the first helicase identified (to our knowledge) with selectivity for (CNG)$_n$ DNA, other than complex CGG repeat structures (44). It is noteworthy that Srs2, not the RecQ homolog Sgs1, exhibits this specificity.

The ~3-fold rate advantage of Srs2 over Sgs1 and UvrD was easily discernible for the proximal (CTG)$_4$-(CTG)$_3$ duplex (Fig. 3B). The differences were also evident for longer CTG duplexes (Fig. 5) and for the intramolecular hairpin (Fig. 6) (see supplemental graphs). These observations suggest that Srs2 more easily overcomes the barrier to unwinding imposed by (CTG)$_n$ DNA over the range where $n = 5$ to 15. Nonetheless, even Srs2 is slowed by the longer substrates. This biochemical feature correlates with the genetics, which showed that the magnitude of protection afforded by Srs2 was lower for longer (CTG) repeat tracts (29). Superior unwinding by Srs2 was also seen for both (CAG)$_n$-(CAG)$_3$ and (CCG)$_3$-(CCG)$_3$ partial duplexes (Fig. 7), again consistent with genetic data (29) (supplemental data). However, the enzyme was less effective on these substrates than on comparable molecules with CTG repeats. This finding is a bit surprising, because the observed thermodynamic stability of hairpins with these sequences is CTG $\rightarrow$ CAG $\rightarrow$ CGG (7, 40). In other words, the structure predicted to be most thermodynamically stable turns out to be the easiest for Srs2 to unwind. Because UvrD and Sgs1 showed a similar pattern, we presume that either there is a sequence preference common to all three helicases or that subtle structural differences in our test molecules provide easier access for the helicases when CTG repeats are present. The slow unwinding of the CCG repeat-containing substrate may also have something to do with its high G+C content. Nonetheless, experiments intended to mimic structural or sequence specificity with the non-CNG repeat or the ATT repeat substrate (Fig. 8) did not recapitulate faster unwinding by Srs2. Instead, the relative helicase activity more closely resembled that seen with the control substrate containing Watson-Crick pairs (Fig. 3C). Thus, the repeating nature of the CNG tract is important in determining biochemical selectivity of Srs2, not the base composition and presence of mismatches.

One interesting clue as to DNA features that determine unwinding selectivity came from experiments where a (CTG)$_3$-(CTG)$_3$ sequence was placed either proximally, centrally, or distally. Proximal placement (Fig. 3B) created a barrier to Sgs1 and UvrD, but not Srs2. However, the unwinding advantage of Srs2 was negated when this CNG repeat was located either centrally or distally (Fig. 9). This suggests that significant modulation of helicase activity is afforded by the duplex sequences that the enzyme encounters immediately as it translocates from the single-stranded tail.

Srs2 joins FEN-1 and Msh2 as proteins with direct influence on triplet repeat expansions. It is equally interesting to contrast the three. FEN-1, like Srs2, helps prevent expansions (9–13), but FEN-1 does so as a nuclease acting at $3'$-flaps (8, 11, 13). Another difference is that FEN-1 is also anti-mutagenic at other DNA sequences (17). Msh2 acts in a different way at triplet repeats. Msh2 promotes, not prevents, expansions in transgenic mouse models (18–21), in part because Msh2-Msh3 binds to hairpins and helps preserve their structure (28). Srs2 likely acts as a helicase to unwind triplet repeat hairpins before they are converted to full expansion mutations. Thus, at least three biochemical activities, flap removal, hairpin binding, and hairpin unwinding, act directly at triplet repeat DNA to influence expansions. However, unlike FEN-1 and Msh2, Srs2 is only known to prevent CNG repeat expansions. srs2 mutants in yeast show no effect on CTG repeat contractions, on dinucleotide repeat mutations, or in increasing the forward mutation rate at CAN1 (29). This unique mutator signature makes Srs2 the first member of a new category of proteins with a strong selectivity for blocking triplet repeat expansions.

In summary, this study clearly indicates that Srs2 is a potent helicase for DNA substrates containing CNG repeats. The correlation of the biochemical and genetic findings suggests the helicase function of Srs2 explains part of its ability to help block expansions in yeast. Other factors probably play additional roles in making Srs2 particularly effective at triplet repeats. For example, Srs2 might be recruited (possibly by protein-protein interactions) to sites where CNG hairpins are likely to form, such as replication forks or repair foci. Another outcome of this study is further support for the idea that triplet repeat hairpins are the mutagenic precursor to expansions. The ability of a helicase like Srs2 to unwind a hairpin might make the triplet repeat sequence accessible for nucleosome removal or for proper reannealing to its complementary strand. More investigation is needed to thoroughly understand the biological role of Srs2 in preventing expansions. However, the results presented in this study strongly imply a direct mechanistic role of Srs2 in DNA metabolism related to triplet repeat expansion.

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