The Helicase Activity of Ribonuclease R Is Essential for Efficient Nuclease Activity*

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Background: Escherichia coli RNase R contains an intrinsic RNA helicase activity.
Results: Walker A and Walker B motifs located in the C- and N-terminal regions, respectively, are required for helicase activity.
Conclusion: RNase R helicase activity utilizes the nuclease catalytic channel and stimulates the nuclease activity.
Significance: These findings help to explain how RNase R efficiently degrades structured RNA.

RNase R, which belongs to the RNB family of enzymes, is a 3’ to 5’ hydrolytic exoribonuclease able to digest highly structured RNA. It was previously reported that RNase R possesses an intrinsic helicase activity that is independent of its ribonuclease activity. However, the properties of this helicase activity and its relationship to the ribonuclease activity were not clear. Here, we show that helicase activity is dependent on ATP and have identified ATP-binding Walker A and Walker B motifs that are present in Escherichia coli RNase R and in 88% of mesophilic bacterial genera analyzed, but absent from thermophilic bacteria. We also show by mutational analysis that both of these motifs are required for helicase activity. Interestingly, the Walker A motif is located in the C-terminal region of RNase R, whereas the Walker B motif is in its N-terminal region implying that the two parts of the protein must come together to generate a functional ATP-binding site. Direct measurement of ATP binding confirmed that ATP binds only when double-stranded RNA is present. Detailed analysis of the helicase activity revealed that ATP hydrolysis is not required because both adenosine 5’-O-(thiotriphosphate) and adenosine 5’-(β,γ-imino)triphosphate can stimulate helicase activity, as can other nucleoside triphosphates. Although the nuclease activity of RNase R is not needed for its helicase activity, the helicase activity is important for effective nuclease activity against a dsRNA substrate, particularly at lower temperatures and with more stable duplexes. Moreover, competition experiments and mutational analysis revealed that the helicase activity utilizes the same catalytic channel as the nuclease activity. These findings indicate that the helicase activity plays an essential role in the catalytic efficiency of RNase R.

RNase R is a ubiquitous 3’ to 5’ exoribonuclease that, by itself, is able to digest RNA molecules with extensive secondary structure (1–4). This nuclease is primarily a degradative enzyme that processively acts on mRNAs and under certain conditions, on rRNA as well (1, 5). However, recent work has found that RNase R also participates in 3’ maturation of 16S rRNA (6). RNase R is relatively large (99 kDa) for a nuclease, but both its N-terminal and C-terminal regions have been found to participate in regulatory processes that effect its stability and cellular localization (7–9). Moreover, as part of the regulatory process that renders it highly unstable, RNase R is post-translationally modified by a single acetyl group (8) that sets in motion its degradation by specific cellular proteases (9). The many unusual properties of RNase R have made it a subject of particular scrutiny among the many known cellular RNases.

Of particular interest is the ability of RNase R to digest structured RNAs in the absence of any added RNA helicase (3, 4), especially because RNase R and polynucleotide phosphorylase appear to be the primary nucleases that degrade structured RNAs in vivo (1, 10). For polynucleotide phosphorylase, this degradation is largely carried out in association with an RNA helicase, as part of the RNA degradosome (11–13). However, for RNase R, its mechanism of structured RNA degradation is not fully understood. In earlier work, our laboratory suggested that it was due to natural thermal breathing and tight binding of the RNA substrate at the bottom of the catalytic channel within the nuclease domain. We hypothesized that such binding forced the substrate to translocate by one nucleotide every catalytic cycle and concomitantly opened one base pair as the enzyme digested through the structured region of RNA (3, 4).

RNase R is a multidomain protein containing two cold shock domains in its N-terminal region, an S1 domain in its C-terminal region, and a central nuclease domain with the intrinsic ability to degrade structured RNAs, although relatively inefficiently compared with the full-length protein (2–4). Thus, other regions of the protein must also contribute to digestion of structured RNA. Other studies revealed that RNase R could complement the essentiality of CsdA, a DEAD-box helicase, at low temperatures (14), and that RNase R also has intrinsic RNA helicase activity (15). Cold shock domain 2 (residues 151–216) was found to be important for both of these activities, but not for the nuclease activity of RNase R. Moreover, nuclease activity was not required for the helicase activity or for its ability to complement the cold shock function of CsdA. Based on these findings, it was concluded that the helicase and nuclease activities of RNase R are independent (15). However, the nature and mechanism of the helicase activity have remained a mystery because the enzyme lacks the sequence and structural signatures seen in helicases.
Here, we present a detailed examination of the helicase activity of RNase R. By a careful motif search, we were able to identify Walker A and B motifs, suggestive of an ATP-binding P-loop domain, and found that both motifs are necessary for ATP binding and helicase activity. Interestingly, the Walker A motif is located in the C-terminal region of RNase R, whereas the Walker B motif is in the CSD2 domain in the N-terminal region indicating that the two parts of the protein must come together to generate the ATP-binding site. We also find that although helicase activity is dependent on binding of ATP, hydrolysis of ATP is not required. Although the nuclease activity of RNase R is not needed for helicase activity, the helicase activity is important for nuclease activity, particularly as the assay temperature is lowered. Mutation of either Walker motif or the absence of ATP eliminates stimulation of the nuclease activity, confirming the role of each motif in the helicase activity. Competition experiments and mutational analysis strongly suggest that both the helicase and nuclease activities are carried out in the same catalytic channel. These findings indicate that the helicase activity plays an important role in the catalytic properties of RNase R.

Characterization of Helicase Activity of RNase R

Here, we present a detailed examination of the helicase activity of RNase R. By a careful motif search, we were able to identify Walker A and B motifs, suggestive of an ATP-binding P-loop domain, and found that both motifs are necessary for ATP binding and helicase activity. Interestingly, the Walker A motif is located in the C-terminal region of RNase R, whereas the Walker B motif is in the CSD2 domain in the N-terminal region indicating that the two parts of the protein must come together to generate the ATP-binding site. We also find that although helicase activity is dependent on binding of ATP, hydrolysis of ATP is not required. Although the nuclease activity of RNase R is not needed for helicase activity, the helicase activity is important for nuclease activity, particularly as the assay temperature is lowered. Mutation of either Walker motif or the absence of ATP eliminates stimulation of the nuclease activity, confirming the role of each motif in the helicase activity. Competition experiments and mutational analysis strongly suggest that both the helicase and nuclease activities are carried out in the same catalytic channel. These findings indicate that the helicase activity plays an important role in the catalytic properties of RNase R.

Materials—Mutagenic primers were synthesized and purified by Sigma Genosys. KOD Hot Start DNA Polymerase was from Novagen. DpnI and bacteriophage T4 polynucleotide kinase were purchased from New England Biolabs, Inc. Protein assay dye reagent concentrate for Bradford assays was obtained from Bio-Rad Laboratories. RNA oligonucleotides were synthesized by Sigma Genosys. [γ-32P]ATP was from PerkinElmer Life Sciences. BugBuster Protein Extraction Reagent was from Novagen. SequaGel for denaturing urea-polyacrylamide gels was from National Diagnostics. The Affi-Gel Blue column was purchased from GE Healthcare. All chemicals were reagent grade.

Cloning of RNase R Mutant Constructs—pET44R(D272N), pET44R(K736A), and pET44R(D168A,D169A) were constructed by standard site-directed mutagenesis of pET44R using the corresponding primer pairs listed in Table 1 (2).

| Mutant primer       | Primer sequence                                      |
|---------------------|------------------------------------------------------|
| D272N               | F: 5’-CCG CTC GCC ACC ATT AAT GCC GAA GAC CCC GCT GAT TTT-3’ |
| K736A               | R: 5’-AAA GTC ACG GGC TCC ACC ATT AAT GGT GAC CAG CGG-3’  |
| D168A,D169A         | F: 5’-CCG CTC GCC ACC GTT CTT GCC ACC CTC GGC GCC GAG AAA GCG AAA-3’ |
| D272N,D168A,D169A   | R: 5’-TATGACTTTCCGAGTTCGCCACGCC-3’                  |

The resulting lysate was centrifuged at 21,000 × g for 30 min at 4°C. The supernatant fraction was applied to an Affi-Gel Blue column, and the column was washed with 2 column volumes of 20 mM HEPES (pH 7.5), 500 mM KCl, 0.5 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 5 units of DNase I/g of wet weight of cells. Cells were disrupted by addition of 0.5 ml of 10× BugBuster reagent and incubation for 20 min at room temperature with shaking. The resulting lysate was centrifuged at 37°C with shaking to an A600 of ~0.6 in 500 ml of yeast/trypton medium supplemented with 100 μg/ml of ampicillin, 34 μg/ml of chloramphenicol, 25 μg/ml of kanamycin, and 10 μg/ml of tetracycline. Expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. Cells were grown for a further 3 h at 37°C. Cells were then harvested by centrifugation at 10,000 × g for 10 min at 4°C, and the resulting cell pellet was stored at −80°C.

Purification of RNase R Mutant Proteins—Full-length wild-type RNase R and RNase R mutant proteins were purified from over-expressing cells as described previously (2) with some modifications. The frozen cell pellet was thawed on ice and resuspended in 4.5 ml of 20 mM HEPES (pH 7.5), 500 mM KCl, 0.5 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 5 units of DNase I/g of wet weight of cells. Cells were disrupted by addition of 0.5 ml of 10× BugBuster reagent and incubation for 20 min at room temperature with shaking. The resulting lysate was centrifuged at 21,000 × g for 30 min at 4°C. The supernatant fraction was applied to an Affi-Gel Blue column, and the column was washed with 2 column volumes of 20 mM HEPES (pH 7.5), 500 mM KCl, 0.5 mM EDTA, 1 mM DTT, and 0.1 mM PMSF. RNase R or RNase R mutant proteins were eluted from the column with 20 mM HEPES (pH 7.5), 1 mM KCl, 0.5 mM EDTA, 1 mM DTT, and 0.1 mM PMSF. Fractions containing RNase R were pooled, divided into aliquots, and stored frozen at −80°C. Although this purification procedure is shortened from that reported previously, based on SDS-PAGE, it leads to wild type and mutant proteins that are each ~90% pure.

Preparation of Oligoribonucleotide Substrates—Oligoribonucleotides were deprotected according to the manufacturer’s instructions. The oligoribonucleotides used were A17, U12C5,A 29G5,U 7C10, and A24G10. Oligoribonucleotides were 5’ labeled with [32P]ATP using T4 polynucleotide kinase and [γ-32P]ATP. A double-stranded helicase substrate consisting of a 17-base pair duplex with a 17-nucleotide 3’ overhang (ds17-32P) was prepared by mixing 5’-ss17-A17 with a [32P] complementary ss17 oligoribonucleotide. For nuclease assays the [32P] label was on the 5’ end of the longer strand. Strands were mixed in a 1:1.2 molar ratio (unlabeled strand in excess) in the presence of 30 mM Tris-HCl (pH 8.0) and 20 mM KCl, heating the mixture in a boiling water bath for 5 min, and then allowing the solution to cool slowly to room temperature.

RNase R Activity Assay—RNase R assays were carried out in 30 μl of reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 300 mM KCl, 0.25 mM MgCl2, 5 mM DTT, and 10 μM oligoribonucleotide substrate. The amount of purified enzyme was as indicated in the respective figure legends. Reaction mixtures
were incubated at the indicated temperatures, portions were taken at the indicated times or at regular intervals for determination of initial rates, and the reaction was terminated by the addition of 2 volumes of gel loading buffer (95% formamide, 20 mM EDTA, 0.025% broomphenol blue, and 0.025% xylene cyanol). Reaction products were resolved on denaturing 7.5% urea, 20% polyacrylamide gels followed by autoradiography. Quantification was carried out using ImageJ (NIH) (16).

**Helicase Assay**—The RNA substrate used to characterize the helicase activity of RNase R was a 17-base pair duplex with a 17-nucleotide 3’ overhang (ds17-A17), and was prepared by mixing 5’-32P-labeled U12-C5 with the non-radioactive complementary oligoribonucleotide G5-A29, as described earlier. The unwinding activity of RNase R was assessed in 30-μl reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 0.5 mM MnCl2, 20 mM NaCl, 2 mM ATP, and 0.1 units of RNase R D272N and varying amounts of double-stranded RNA substrate. Reaction products were analyzed on a 20% native Tris borate-EDTA polyacrylamide gel followed by autoradiography.

**Filter Binding Assays**—The double-filter nucleic acid binding assay developed by Wong and Lohman (17) as adapted by Tanaka and Schwer (18) was used. Briefly, nitrocellulose membranes were presoaked in 0.5 M KOH for 10 min and then rinsed in H2O until the pH returned to neutral. Biodyne Plus nylon membranes were washed once in 0.1 M EDTA (pH 8.0) for 10 min, 3 times in 1 M KCl for 10 min each followed by rinsing in 0.5 M KOH for 1 min, and finally rinsed with H2O until the pH returned to neutral. Nitrocellulose and nylon membranes were equilibrated in binding buffer (50 mM Tris-Cl (pH 7.5), 50 mM MgCl2, 20 mM NaCl) at 4 °C for at least 1 h before use. Fifty μl of reaction mixtures containing 50 mM Tris-Cl (pH 7.5), 50 mM MgCl2, 20 mM NaCl, 2 mM ATP, 0.01 μCi/μl of [γ-32P]ATP, 0.1 μM RNase R D272N and varying amounts of double-stranded or single-stranded RNA substrate as indicated were incubated for 30 min at 37 °C. A 96-well dot-blot apparatus (Bio-Rad) was used. Typically, 0.5 units of RNase R were incubated at the indicated temperatures, portions were taken at the indicated times or at regular intervals for determination of initial rates, and the reaction was terminated by the addition of 2 volumes of gel loading buffer (95% formamide, 20 mM EDTA, 0.025% broomphenol blue, and 0.025% xylene cyanol). Reaction products were resolved on denaturing 7.5% urea, 20% polyacrylamide gels followed by autoradiography. Quantification was carried out using ImageJ (NIH) (16).

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**Identification of the ATP-binding Site**—Sequence analysis indicated that RNase R lacks the multiple motifs usually associated with RNA helicases. However, because RNase R helicase activity is ATP dependent, there must be an ATP-binding site. To identify this site, RNase R sequences were aligned with the Walker A and Walker B motifs (19, 20), and we also identified a Walker A-like sequence (FVVPDD) near the C-terminal region of the protein in RNase R. Interestingly, whereas the Walker A motif is located in the C-terminal region of RNase R, the putative Walker B motif is in the CSD 2 domain in the N-terminal region (Fig. 1A), suggesting that the N- and C-terminal regions of the protein must come together to generate the ATP-binding site.

2 The abbreviations used are: ATPγS, adenosine 5’-O-(thiotriphosphate); AMP-PNP, adenosine 5’-(β,γ-imino)triphosphate.
Characterization of Helicase Activity of RNase R

Bioinformatic analysis of RNase R sequences from many diverse organisms using the UniProt database revealed that the ATP binding domain, consisting of both Walker A and Walker B motifs, is conserved in many different genera of mesophilic bacteria. We found that 88% of the 56 mesophilic bacterial genera analyzed encompassing thousands of species contained both Walker A and Walker B motifs, and in each case the A and B motifs were located in the C- and N-terminal regions, respectively. On the other hand, of 26 thermophilic bacterial genera examined, none contained an ATP-binding domain. These findings suggest that the role of the helicase activity may be most important at lower temperatures, and this was confirmed experimentally (see below).

Mutational Analysis of the ATP-binding Motifs—To confirm that the putative Walker A and Walker B motifs identified by sequence analysis actually function in that capacity, we mutated RNase R to create mutant proteins carrying single amino acid substitutions in the Walker A and Walker B motifs. Assays were carried out as described under "Experimental Procedures" with 2.5 nM [53H]/11032-32P]U12C5:G5A29 substrate (shown on left) and 1 μM enzyme. B, effect of ATP concentration. Activity was measured using 1 μM RNase R D272N as in panel A at the indicated ATP concentrations for 30 min. C is a no enzyme control incubated for 30 min. Shown is a representative experiment carried out three times with essentially identical results.

FIGURE 1. A, schematic showing the domain organization of E. coli RNase R. The three RNA-binding domains CSD1, CSD2, and S1, the catalytic nuclease domain, and the C-terminal basic domain are shown. The position of the Walker A and Walker B motifs are noted. B, sequence alignment using UniProt of available RNase R sequences from 49 different genera of mesophilic bacteria that show conservation of the ATP-binding Walker A and Walker B motifs, highlighted in yellow.

FIGURE 2. Helicase activity of RNase R. A, effect of ATP on helicase activity of RNase R and RNase R mutant proteins. Assays were carried out as described under "Experimental Procedures" with 2.5 nM [53H]/11032-32P]U12C5:G5A29 substrate (shown on left) and 1 μM enzyme. B, effect of ATP concentration. Activity was measured using 1 μM RNase R D272N as in panel A at the indicated ATP concentrations for 30 min. C is a no enzyme control incubated for 30 min. Shown is a representative experiment carried out three times with essentially identical results.
conserved amino acid residues in each of the motifs (K736A for Walker A and D168A,D169A for Walker B), and analyzed the helicase activity of RNase R. As shown in Fig. 2A, mutation of either the Walker A or B motifs eliminated RNA helicase activity confirming the essential role of these residues.

Role of the Helicase Activity in the Exoribonuclease Activity of RNase R—The presence of an intrinsic RNA helicase activity in RNase R raised the obvious question of how it relates to the nuclease activity of RNase R. To examine this point, we made use of the K736A and D168A,D169A mutant proteins that lack nuclease activity of RNase R. To examine this point, we made use of the K736A and D168A,D169A mutant proteins that lack nuclease activity of RNase R. As shown in Fig. 4A, the absence of helicase activity had no effect on the ability of RNase R to degrade a single-stranded, 34-nucleotide long substrate. Neither the rate of RNA degradation nor the limit products generated were altered with the mutant proteins as compared with wild type RNase R. Likewise, using wild type RNase R, there was no effect of eliminating ATP on the ability of the enzyme to degrade the single-stranded RNA substrate at any temperature between 37 and 10 °C (Fig. 4B). These data strongly suggest that the helicase activity does not influence degradation of single-stranded RNA.

On the other hand, there was a dramatic effect of the helicase activity when the substrate was a double-stranded RNA (ds17-A17) (Fig. 5). Thus, as shown in Fig. 5A, ATP stimulated the nuclease activity of wild type RNase R, and the stimulation became more pronounced as the assay temperature was reduced in steps from 37 to 10 °C. In contrast, ATP had no effect with the K736A helicase-deficient mutant protein (Fig. 5B). Likewise, ATP had no effect with the D168A,D169A mutant protein (data not shown). Interestingly, the ATP analogs, AMP-PNP and ATPγS, stimulated the wild type nuclease activity as well as ATP at all temperatures (data not shown), confirming that they are able to function as cofactors for the helicase activity, as shown in Fig. 2.

To further analyze the importance of the helicase activity, we used a double-stranded substrate in which 10 of the 17 base pairs were consecutive G-C pairs. With this substrate, the effect of added ATP was even more pronounced, stimulating activity close to 4-fold at 20 °C (Fig. 5C). These findings indicate that the helicase activity is an important, intrinsic component of the nuclease activity of RNase R, and that its influence becomes more pronounced at lower temperatures and with GC-rich RNAs.

Double-stranded RNA Stimulates ATP Binding—Inasmuch as the ATP-binding site contains motifs widely separated in the amino acid sequence of RNase R, it was of considerable interest to understand what conditions promote their coming together to generate a functional binding site. To examine this question, we utilized a filter binding assay to quantify the amount of [$^{32}$P]ATP bound upon addition of single-stranded or double-stranded RNA (Fig. 6). Essentially no RNase R molecules (<3%) contained bound ATP in the absence of RNA. Likewise, very little ATP was bound in the presence of single-stranded RNA. In contrast, increasing amounts of double-stranded RNA induced ATP binding such that almost all the RNase R molecules ultimately contained bound ATP. These data indicate that the ATP-binding site does not pre-exist in the absence of RNA, and that duplex RNA promotes the joining of the separated Walker A and Walker B motifs to generate a functional binding site.

Helicase Activity of RNase R Utilizes the Nuclease Catalytic Channel—To further analyze the relationship between the nuclease and helicase activities of RNase R, we inquired whether the substrate binding channel known to be used for the exoribonuclease activity (4) was also required for the helicase activity.

To examine this question, we measured the helicase activity of the RNase R D272N nuclease mutant protein in the presence of different concentrations of A4 or A17, known substrates of the nuclease activity, that should bind and occupy the channel (3). As shown in Fig. 7A, the helicase activity of RNase R decreased in steps from 37 to 10 °C. In contrast, ATP had no effect with the K736A helicase-deficient mutant protein (Fig. 7B). Likewise, ATP had no effect with the D168A,D169A mutant protein (data not shown). Interestingly, the ATP analogs, AMP-PNP and ATPγS, stimulated the wild type nuclease activity as well as ATP at all temperatures (data not shown), confirming that they are able to function as cofactors for the helicase activity, as shown in Fig. 2.
R was strongly inhibited by increasing concentrations of either A4 or A17. These data strongly suggest that the helicase activity utilizes the same catalytic channel as the nuclease activity.

To obtain additional evidence for this conclusion, we made use of several channel mutants. Previous work from our laboratory showed that RNase R channel mutants R572K and R572K,H456N,H565T resulted in weaker substrate binding within the nuclease domain channel and that these mutant proteins had difficulty in degrading structured RNA (4). These mutations were combined with the D272N nuclease mutant, and helicase activity was measured. As shown in Fig. 7B, the helicase activity of the RNase R channel mutants R572K and R572K,H456N,H565T decreased 35 and 46%, respectively, compared with the helicase activity of RNase R D272N alone. These findings support the conclusion that the helicase activity of RNase R uses the same catalytic channel as utilized for the nuclease activity.

Discussion
RNase R is an unusual exoribonuclease in that it can digest highly structured RNA without an accessory factor (1). The studies described here greatly help in understanding how this is accomplished. It has been known for several years that RNase R contains an intrinsic RNA helicase activity, in addition to its nuclease activity, but it was believed the two activities are independent of each other (15). However, our data show that efficient nuclease activity, particularly at low temperatures and with very stable RNA duplexes requires the helicase activity. Moreover, we find that both activities are carried out within the same nuclease domain catalytic channel. It was also unclear from the earlier work (15) whether the RNA helicase activity requires ATP. This work clearly indicates that ATP is required, but that ATP hydrolysis is not needed. The lack of ATP hydrolysis also has been observed previously with other helicases (21, 22).

The helicase activity present in RNase R is highly unusual in that it lacks almost all the motifs usually associated with RNA helicases, except for those involved with ATP binding, the P-loop Walker A and the Walker B motifs (19, 20). Interestingly, these two motifs are widely separated in the RNase R sequence; Walker A is in the C-terminal basic region and Walker B in the N-terminal CSD2 domain (see Fig. 1A). This raises the obvious question of how these separated motifs come together to generate an ATP-binding site and where this site is located in the RNase R structure. Although no structure is available for E. coli RNase R, important clues to the disposition of the Walker A and Walker B motifs can be gleaned from the structure of the closely related RNase II (23, 24). RNase II and RNase R are close homologs (26.4% identity; 56.7% similarity), and both nucleases share the same sequence motifs and domain structures. Given this homology and the similar architectures of other RNase R family enzymes such as Rrp44 (25, 26), the structure of the core of RNase R is likely to be very similar to RNase II. In RNase II, the three RNA-binding domains come together to form a funnel shape at the top of a narrow nuclease channel that can only accommodate ssRNA (23). The region corresponding to the Walker B motif (residues 102–103 in...
RNase II would lie tantalizingly close to the inside surface of this RNA binding funnel (Fig. 8A). The position of the Walker A motif is harder to pinpoint because RNase II lacks the C-terminal extension present in RNase R. However, the Walker A motif has to lie just a few residues beyond the C-terminal end of the S1 domain. So, the Walker A motif likely also lies very close to the RNA binding funnel, but at some distance from the Walker B motif (Fig. 8A).

In earlier work (4), prior to the discovery of the helicase activity, our laboratory had already presented a model to explain how RNase R is able to digest structured RNA in the absence of an added energy source. As noted, single-stranded RNA can access the catalytic channel, whereas double-stranded RNA cannot because the channel is too narrow (4). In addition, we had found that single-stranded RNA binds very tightly within the channel. Based on this information, we suggested that when the duplex portion of an RNA substrate approached the channel, the end of the duplex would transiently open by thermal breathing allowing the single-strand end to advance one residue into the channel as one nucleotide was cleaved from the 3' end of the RNA at the bottom of the channel. Re-annealing of RNase II would lie tantalizingly close to the inside surface of this RNA binding funnel (Fig. 8A). The position of the Walker A motif is harder to pinpoint because RNase II lacks the C-terminal extension present in RNase R. However, the Walker A motif has to lie just a few residues beyond the C-terminal end of the S1 domain. So, the Walker A motif likely also lies very close to the RNA binding funnel, but at some distance from the Walker B motif (Fig. 8A).

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the opened base pair would be prevented by a “wedge” located at the top of the channel that bound the displaced strand. This model satisfactorily explained the available data and proposed that RNase R utilized energy from thermal motion and substrate binding to enable it to move through a duplex RNA substrate 1 base pair at a time.

Based on all the new information presented here, we are now able to expand on that model and suggest that the helicase activity, located at the channel entrance, facilitates opening of the RNA duplex (Fig. 8B) and may, in fact, serve as the wedge. Additionally, we propose that the presence of duplex RNA at the entrance to the catalytic channel induces a conformational change that brings the Walker A and B sequences together to generate the ATP-binding site, as confirmed by ATP binding data (Fig. 6). Once bound, the presence of ATP (or other triphosphates) stimulates binding of the strands of the RNA duplex to the CSD and S1 domains that results in partial strand separation. However, in the absence of nuclease activity, strand separation continues and is measured in vitro as helicase activity. The details of how this process occurs are still unclear. During nuclease action, only partial separation of the duplex at the channel entrance would be needed as each cycle of cleavage and

FIGURE 7. Helicase activity utilizes the nuclease catalytic channel. A, helicase activity of RNase R D272N in the presence of single-stranded oligonucleotides. Assays were carried out for 30 min in the presence of different concentrations of A₄ or A₁₇, as indicated. B, helicase activity of RNase R catalytic channel mutants. Helicase assays in both panels were carried out in a reaction mixture containing 2.5 nm RNA substrate, 0.1 μM enzyme, and 2 mM ATP for the indicated times. Left panel, ○, RNase R D272N; □, RNase R D272N,R572K; right panel, ○, RNase R D272N; □, RNase R D272N,H456N,H565T,R572K. Quantification shown is the average of three experiments ± S.D. A representative gel is shown for each experiment.
translocation to the catalytic site would propel the nuclease through the RNA structure (Fig. 8B). Although certain details of this model remain to be verified experimentally, we believe it satisfactorily explains what is currently understood about RNase R action.

Our identification of the Walker A and Walker B motifs does not agree completely with the studies of Awano et al. (15). Those workers showed by deletion analysis that CSD2 is essential for the ability of RNase R to complement the absence of the helicase, CsdA, at low temperature, which agrees with our identification of the Walker B motif within this domain. On the other hand, they found that removal of the C-terminal S1 and basic domains had no effect on the ability of RNase R to complement even though, based on our data, that deletion would have removed the Walker A motif. One possible explanation for this apparent discrepancy between the results is that in their experiments the RNase R constructs were expressed from multicopy plasmids. If RNase R lacking a Walker A motif were still able to bind ATP weakly, perhaps sufficient helicase activity could be generated from the overexpressed RNase R to enable complementation of a CsdA mutant strain. Further work will be needed to completely clarify this point.

Another interesting, related question is, how does RNase R complement a CsdA helicase-deficient strain and avoid degrading the RNA molecule that is being acted upon given that we have found that both the nuclease and helicase activities take

**FIGURE 8.** Structural and schematic model of RNase R based on the closely related RNase II. A, approximate locations of the Walker A and Walker B motifs (highlighted by ovals) shown on the ribbon structure of the closely related RNase II crystallized with an RNA fragment trapped in the nuclease channel (21). The view shown is from the front of the enzyme, with the RNA binding clamp on the top (N-terminal CSD1 and CSD2 domains in *pink* and *blue*, and the C-terminal S1 domain in *red*). These three domains form an open-ended RNA-binding funnel that leads into the narrow channel in the nuclease domain (shown in *green*). The RNA strand is shown in a stick representation, and one of the Asp residues in Walker B (D102 in RNase II) can be seen as *blue* sticks at the bottom of the oval. B, schematic model of single-strand and double-strand RNA bound on RNase R to explain enhancement of nuclease activity in the presence of ATP. The RNA-binding domains are shown in *pink*, *blue*, and *red* for the CSD1, CSD2, and S1 domains, respectively. The nuclease domain is in *green* with its catalytic center highlighted by a *pink* arrow. No change in structure occurs upon single-strand RNA binding. Upon double-strand RNA binding, the Walker A and Walker B motifs come close to each other to generate an ATP-binding site. ATP binding stimulates strand separation, likely due to a conformational change in the protein. The newly separated strand moves forward into the nuclease channel to position the next nucleotide for cleavage at the 3′-end. The color coding in panels A and B is the same.
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place in the same catalytic channel? Although we do not yet have a definitive answer, we suspect that it may be due to physical separation of the two activities. The helicase activity needs to be located at the entrance to the catalytic channel to facilitate partial separation of the RNA duplex strand to allow the 3′ terminal strand to enter the catalytic channel. In contrast, the nuclease catalytic site is located at the bottom of the channel, ~5 nucleotides away (2). Thus, it would be possible for an RNA molecule to be acted on by the helicase activity and avoid degradation if it does not penetrate too deeply into the channel. Exactly how this might be accomplished remains to be determined.

Most importantly, the findings presented here provide an explanation for why RNase R has an intrinsic helicase activity (15). Our data show that even though RNase R can degrade RNA in the absence of the helicase at temperatures as low as 10 °C, and even when an RNA duplex is GC-rich, the associated RNA helicase activity greatly stimulates this basal nuclease activity. Moreover, the fact that similarly located Walker A and Walker B motifs are found in many other bacterial genera strongly suggests that the RNA helicase is an important component of the RNase R molecule. We anticipate that further studies will continue to illuminate the mechanism by which RNase R degrades structured RNAs.

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