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RNase R mutants elucidate the catalysis of structured RNA: RNA-binding Domains select the RNAs targeted for degradation.

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Abbreviations: 3D, three-dimensional; CSD, cold shock domain; RNB, RNase II catalytic domain; HTH, helix-turn-helix; IPTG, isopropyl-(3-D-thiogalactoside); DTT, dithiotreitol; PMSF, phenylmethylsulfonyl fluoride; SPR, Surface Plasmon Resonance; K_D , dissociation constant.

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Synopsis

The RNase II super family is an ubiquitous family of exoribonucleases essential for RNA metabolism. RNase II and RNase R degrade RNA in the 3' to 5' direction in a processive and sequence independent manner. However, while RNase R is capable of degrading highly structured RNAs, the RNase II activity is impaired by the presence of secondary structures. RNase II and RNase R share structural properties and have a similar modular domain organization. The eukaryotic RNase II homologue, Rrp44/Dis3, is the catalytic subunit of the exosome, one of the most important protein complexes involved in the maintenance of the correct levels of cellular RNAs. In this work we constructed truncated RNase II and RNase R proteins and point mutants and characterized them regarding their exoribonucleolytic activity and RNA-binding ability. We report that Asp-280 is crucial for RNase R activity without affecting RNA binding. When Tyr-324 was changed to alanine the final product changed from 2 to 5nts showing that this residue is responsible for setting the end-product. We have shown that the RNB domain of RNase II has catalytic activity. The most striking result is that the RNase R RNB domain *per se* degrades double stranded substrates even in the absence of a 3'-overhang. Moreover, we demonstrate for the first time that the substrate recognition of RNase R depends on the RNA binding domains that target the degradation of RNAs that are "tagged" by a 3'-tail. These results can have important implications for the study of poly(A)-dependent RNA degradation mechanisms.

Introduction

E. coli RNase II is the prototype of the RNase II super family of exoribonucleases. Homologues of RNase II/R are present in all domains of life [1-4]. RNase R is a member of this family that is involved in mRNA degradation, in RNA and protein quality control and has been shown to be required for virulence [5-9]. In the nucleus and cytoplasm of eukaryotic cells, the RNase II homologue Rrp44/Dis3 is a subunit of the exosome, an essential multiprotein complex, involved in the processing, turnover and quality control of different types of RNAs [3]. Rrp44/Dis3 is the only catalytically active nuclease in the yeast core exosome [10] and recently it was shown that it contains both exo and endoribonuclease activity [11, 12].

RNase II and RNase R share catalytic properties: they both degrade RNA processively, in the 3' to 5' direction releasing 5'-nucleotide monophosphates. These enzymes also share structural properties, including 60% sequence homology [13]. Their activity is sequence independent but while RNase II is sensitive to secondary structures, RNase R is capable of degrading highly structured RNA [6, 9, 13, 14]. Another difference is that the final degradation product of RNase II is a 4 nucleotide fragment, whereas the end-product of RNase R is a 2 nucleotide fragment [14-16].

RNase R is a 92 KDa protein encoded by the *rnr* gene. It is involved in the degradation of different types of RNAs such as rRNAs, sRNAs and mRNAs. It was shown that RNase R has *in vivo* affinity for polyadenylated RNA and that it can be a key enzyme involved in poly(A) metabolism [17]. RNase R is the only known 3' to 5' exoribonuclease able to degrade through double stranded RNA without the aid of helicase activity [18]. It is a cold shock protein that is regulated transcriptionally and post-transcriptionally [7, 8]. The activity of RNase R is modulated according the growth conditions of the cell [19] and its levels increase in stationary phase and under stress conditions [6, 8, 9]. It has been shown that this protein is also involved in pathogenesis in different microorganisms [5, 20-22].

RNase II is a 72 KDa protein encoded by gene *rnb*. In *E. coli*, this protein is the major hydrolytic enzyme that is responsible for 90% of the exoribonucleolytic activity in crude extracts [23]. RNase II expression is differentially regulated at the transcriptional and post-transcriptional levels and the protein can be regulated by the environmental conditions [19, 24, 25]. The determination of the three-dimensional structure of *E. coli* RNase II showed that RNase II consists of 4 domains: two N-terminal cold shock domains (CSD1 and CSD2), one central RNB catalytic domain, and one C-terminal S1 domain [26, 27] (Figure 1). The structural and biochemical analysis helped explaining the activity of the enzyme and led to the proposal of a model for RNA degradation by RNase II. RNA contacts RNase II in two different and non-contiguous regions, the anchoring and the catalytic regions [16, 26]. The shortest RNA substrate able to retain contacts with these two regions is a 10-nucleotide fragment and it was known that this was the minimum size necessary to maintain the processivity of the enzyme [16, 26, 28]. It was demonstrated that Tyr-313 and Glu390 are important to the discrimination of cleavage of RNA *versus* DNA [29]. During the determination of key residues for catalysis, we have recently discovered that the substitution of the Glu-542 by alanine leads to a 100-fold increase in the exoribonucleolytic activity, turning RNase II into a "super-enzyme" [29].

The structure of the RNA bound complex allowed explaining why 4nt is the final degradation product for RNase II [26]. This is a result of the tight packing of the five 3'-terminal nucleotides in the catalytic cavity, mediated by the aromatic residues Tyr-253 and Phe-358 [26]. It was reported that the highly conserved residue Tyr-253 is the one responsible for setting the end-product of RNase II and it is extremely important for the maintenance of the "RNA clamping" in the catalytic site of RNase II [28].

The structural model of *E. coli* RNase R protein was constructed based on the RNase II structure; the two enzymes share a common 3D arrangement, being all the critical residues for exoribonucleolytic activity located in equivalent spatial positions [28]. By comparing the protein models it was noteworthy that these two proteins have a common arrangement of the clamping tyrosine amino acid but, in contrast, the presence of Phe-358 is exclusive to RNase II. RNase R protein presents a Phe residue in the immediate downstream position (Phe-429), perhaps with a similar functionality in fixing the RNA. The differences in the equivalent amino acid Phe-358 in RNase II and RNase R could explain their differences in regard to RNA degradation [28].

It was also shown that the conserved Asp-209 residue is directly involved in RNA cleavage by RNase II. This is the only critical aspartate for RNase II activity [28, 30], and its substitution by Asn was responsible for the total loss of the protein activity without affecting its RNA binding ability [30]. Moreover, a similar mutation in the yeast RNase II homologue Rrp44/Dis3 (D551N) totally abolished activity without reducing substrate binding. This mutation was also responsible for a very strong growth defect, suggesting that the phenotype of this mutant is very important for yeast physiology [10, 31].

In this work we constructed and analyzed the equivalent mutants to Tyr253Ala and Asp209Asn of *E. coli* RNase II in RNase R, Tyr324Ala and Asp280Asn, respectively. Our aim was to confirm their importance in the activity of the enzymes of the RNase II family. We observed that the mutant Asp280Asn has no activity but is still able to bind RNA efficiently, which confirms the importance of this aspartate in catalysis. When Tyr-324 was changed into an alanine, we verified that the final product of RNase R changed from 2 to 5 nts, confirming the importance of this residue in setting the final-end product in this family of enzymes.

Furthermore, we constructed a set of truncated proteins of RNase R lacking CSD and/or S1 domains in order to understand the contribution of each domain (Figure 1). We analyzed the activity and the binding ability of the truncated proteins using different substrates and observed that the RNB domain of RNase R alone is capable of degrading RNA molecules. In addition, we show that this domain is sufficient for the degradation of structured substrates, and that is also able to bind to RNA, although with less efficiency when compared with the wild type protein. We also observed that the CSD and S1 domains are responsible for the binding of the proteins to the substrate and that they are crucial for the recognition of which substrates are targeted to be degraded. In the absence of both these domains the protein is capable of degrading perfect double stranded RNAs that lack the 3' end overhang. To date it had always been reported that the existence of a 3' end overhang of at least 5 nts length for RNase R was essential for catalysis to occur; here we unravel the reason why.

Experimental

Restriction enzymes, T4 DNA ligase, Pfu DNA polymerase and T4 Polynucleotide Kinase were purchased from Fermentas, Unlabeled oligonucleotide primers were synthesized by STAB Vida, Portugal.

Strains

The *E. coli* strains used were DH5 α (F' *fhuA2* Δ (*argF-lacZ*)U169 *phoA glnV44* Φ 80 Δ (*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17a*) [32] for cloning experiments and BL21(DE3) (F' *r_B⁻ m_B⁻ gal ompT (int::P_{lacUV5} T7 gen1 imm21 nin5*) [33] for expression and purification of enzymes.

Construction of RNase R mutants by PCR Overlapping

The point mutations Asp280Asn and Tyr324Ala were introduced into pABA-RNR (Table 1) by PCR Overlapping. The primers used in the construction of RNase R mutants were Asp280Asn_Fw, Asp280Asn_Rev, Tyr324Ala_Fw and Tyr324Ala_Rev (Table 2). All mutant constructs were confirmed by DNA sequencing at STAB Vida, Portugal.

Construction of truncated proteins

The truncated proteins were constructed by removing different regions of *rnb* and *rnr* genes in pFCT6.1 and pABA-RNR plasmids (Table 1). *XhoI* restriction site was introduced into pFCT6.1 in the position 512 by overlapping PCR using the primers pFCT_XhoI512_Fw and pFCT_XhoI521_Rev (Table 2) originating the pFCT_XhoI521 plasmid. The natural occurrence of an *XhoI* recognition site in the position 69 allowed us to construct the pFCT_ΔCSDs by digesting the plasmid pFCT_XhoI521 with *XhoI* (that originated degradation products with 6955 bps and 456 bps, the last one corresponding to CSD1 and CSD2 of RNase II) and circularizing the fragment with 6955 bps. The protein RNase II_RNB was constructed by introduction of a stop codon (TAA) at the position 1735 in the plasmid pFCT_ΔCSDs using the primers RNB_Stop1735_Fw and RNB_Stop1735_Rev (Table 2). In the pABA-RNR plasmid (Table 1) it was introduced the *NdeI* restriction site in the position 549 by overlapping PCR using the primers RNR_NdeI549_Fw and RNR_NdeI549_Rev (Table 2) originating the pABA-RNR_NdeI549 plasmid. The natural occurrence of an *NdeI* recognition site in the position 60 allowed us to construct the pABA-RNR_ΔCSDs by digesting the plasmid pABA-RNR_NdeI549 with *NdeI* (that originated degradation products with 7624 bps and 651 bps, the last one corresponding to HTH, CSD1 and CSD2 of RNase R) and circularizing the fragment with 7624 bps. The proteins RNR_ΔS1 and pABA-RNR_RNB were constructed by introduction of a stop codon (TAA) at the position 1927 in the plasmids pABA-RNR and pABA-RNR_ΔCSDs, respectively, using the primers RNR_Stop1927_Fw and RNR_Stop1927_Rev (Table 2).

Overexpression and purification of wild type and RNase II mutants

The plasmid used for expression of wild-type *E. coli* histidine-tagged RNase II protein was pFCT6.1 plasmid [19] (Table 1). This plasmid contains the *rnb* gene cloned into pET-15b vector (Novagen) under the control of $\phi 10$ promoter, allowing the expression of the (His)₆-tagged RNase II fusion protein. The plasmid used for expression of wild-type *E. coli* histidine-tagged RNase R protein was pABA-RNR (Table 1) [15] plasmid that contains the *rnr* gene cloned into pET-15b vector (Novagen) under the control of $\phi 10$ promoter, allowing the expression of the (His)₆-tagged RNase R fusion protein.

All plasmids were transformed into BL21(DE3) *E. coli* strain (Novagen) to allow the expression of the recombinant proteins. Cells were grown at 30°C in 100 ml LB medium supplemented with 150 µg/ml ampicillin to an A₆₀₀ of 1.5. Then, they were transferred to 18°C for 30 minutes and then induced by addition of 0.5 mM IPTG; induction proceeded for 20 hours at 18°C. Cell cultures were pelleted by centrifugation and stored at -80°C.

Purification of all proteins was performed by histidine affinity chromatography using HiTrap Chelating HP columns (GE Healthcare) and AKTA HPLC system (GE Healthcare) following the protocol previously described [14, 34]. Briefly, cell suspensions were lysed using a French Press at 9,000 psi in the presence of 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The crude extracts were treated with Benzonase (Sigma) to degrade the nucleic acids and clarified by a 30 min centrifugation at 10,000 g. The clarified extracts were then added to a HiTrap Chelating Sepharose 1 ml column equilibrated in buffer A (20 mM Tris-HCl, 0.5 M NaCl, pH8) plus 20 mM imidazol and 2 mM β-mercaptoethanol. Protein elution was achieved by a continuous imidazol gradient (from 20 mM to 500 mM) in buffer A. The fractions containing

the purified protein were pooled together and buffer exchanged to buffer B (20 mM Tris-HCl pH8, 100 mM KCl and 2 mM β -mercaptoethanol) using a desalting 5 ml column (GE Healthcare). Eluted proteins were concentrated by centrifugation at 15 °C with Amicon Ultra Centrifugal Filter Devices of 30,000 MWCO (Millipore). Protein concentration was determined by spectrophotometry and 50% (v/v) glycerol was added to the final fractions prior storage at -20 °C. 0.5 μ g of each purified protein was applied in a 8% SDS-PAGE and visualized by Coomassie blue staining (data not shown).

Activity assays

Exoribonucleolytic activity was assayed using three different RNA oligoribonucleotides as substrates [14, 34]. The 30mer oligoribonucleotide (5'-CCCGACACCAACCACUAAAAAAAAAAAAAAAAA-3'), the 16mer oligoribonucleotide (5'-CCCGACACCAACCACU-3') and the poly(A) chain of 35nts were labelled at its 5'-end with [γ -³²ATP] and T4 polynucleotide kinase. The RNA oligomers were then purified using Microcon YM-3 Centrifugal Filter Devices (Millipore) to remove the non-incorporated nucleotides. The labelled 30mer and 16mer oligoribonucleotides were hybridized to the complementary 16mer oligodeoxiribonucleotide (5'AGT GGT TGG TGT CGG G 3'), thus obtaining the corresponding double stranded substrate 16-30ds and 16-16ds respectively. The hybridization was performed in a 1:1 (mol:mol) ratio in the Tris component of the activity by 5 min of incubation at 68°C followed by 45 min as room temperature. The exoribonucleolytic reactions were carried out in a final volume of 10 μ l containing 30 nM of substrate, 20 mM Tris-HCl pH8, 100 mM KCl, 1 mM MgCl₂, and 1 mM DTT. The amount of each enzyme added to the reaction is indicated in the respective Figures. Reactions were started by the addition of the enzyme and incubated at 37°C. Samples were withdrawn at the time points indicated in the Figures and the reaction was stopped by adding formamide-containing dye supplemented with 10 mM EDTA. Reaction products were resolved in a 20% polyacrylamide/7 M urea and analyzed by autoradiography. The exoribonucleolytic activity of the enzymes was determined by measuring and quantifying the disappearance of the substrate in several distinct experiments in which the protein concentration was adjusted in order that, under those conditions, less than 25% of substrate was degraded. Each value obtained represents the mean of these independent assays. The exoribonucleolytic activity of the wild-type enzymes was taken as 100%.

Surface plasmon resonance analysis - BIACORE

Biacore SA chips were obtained from Biacore Inc. (GE Healthcare). The Flow cells of the SA streptavidin sensor chip were coated with a low concentration of the following substrates. On flow cell 1 no substrate was added so this cell could be used as the control blank cell. On flow cell 2 a 5' biotinylated 25-nucleotide RNA oligomer (5'-CCC GAC ACC AAC CAC UAA AAA AAA A-3') was added while on flow cell 3 we added a 5' biotinylated 30-mer poly(A) oligomer to allow the study of the protein interaction with different single-stranded RNA substrates. The target substrates were captured on flow cells 2 and 3 by manually injecting 20 μ l of a 500 nM solution of the substrates in 1 M NaCl at a 10 μ l/min flow rate, as described in previous reports [28, 29, 34, 35]. The biosensor assay was run at 4°C in the buffer with 20 mM Tris-HCl pH8, 100 mM KCl, 1 mM DTT and 25 mM EDTA. The proteins were injected over flow cells 1, 2 and 3 for 2 min at concentrations of 10, 20, 30, 40 and 50 nM using a flow rate of 20 μ l/min. All experiments included triple injections of each protein concentration to determine the reproducibility of the signal and control injections to assess the stability of the RNA surface during the experiment. Bound protein was removed with a 60-s wash with 2 M NaCl, which did not damage the substrate surface. Data from flow cell 1 were used to correct for refractive index changes and non-specific binding. Rate constants and equilibrium

constants were calculated using the BIA EVALUATION 3.0 software package, according to the fitting model 1:1 Langmuir Binding.

Multiple sequence alignment.

Homologous sequences belonging to the RNase II family of proteins in protein databases were obtained using Blast [36] and they were aligned using ClustalW [37] and T-COFFEE [38] algorithms.

Results and Discussion

Arg-280 is crucial for RNase R activity without affecting RNA binding ability. The active site of RNase II has four conserved aspartic residues that are the responsible for positioning the RNA substrate correctly in order to promote the nucleophilic attack of the phosphodiester bond [26]. Previous studies have shown that, in this protein, Arg-209 is a crucial residue for the catalytic activity without affecting RNA binding [30] and that this aspartate is the only one that is essential for the activity of RNase II [28]. Since this is a highly conserved residue in this family of enzymes (Supplementary Figure 1), we also mutated the correspondent amino acid in RNase R, Arg-280, to better understand the reaction mechanism of this exoribonuclease. For that, we introduced the point mutation Arg280Asn into the pABA-RNR [15], and overexpressed and purified the respective protein. The exoribonucleolytic activity of this mutant protein was analyzed and compared to the wild type by performing activity assays using different types of RNA substrates.

Wild type RNase R is able to degrade single and double-stranded RNA substrates releasing 2nts fragment as the shortest final end-product in both cases (Figure 2). In fact, the final end-product is a mixture of a 2nts and 4nts fragments (Figure 2). The structure of RNase II showed that in the catalytic pocket 5nts of RNA were clamped between the aromatic residues Tyr-253 and Phe-358 [26]. Tyr253 is highly conserved and equivalent residues are present in all RNase II family members (Supplementary Figure 1). However in RNase R the equivalent residue to Phe-358 does not exist and that could be a possible explanation for the differences regarding the final end-product [28]. In RNase R there is a Phe residue (Phe-429) in the position immediately downstream of the equivalent residue of Phe-358 in RNase II. It is possible that some RNA fragments are still partially “clamped” and therefore they are released when they reach 4nts. Others bind more efficiently the catalytic cavity and are able to be degraded up to a 2nts fragment. This issue will be clarified when the RNase R crystal structure is solved. When the D280N mutant of RNase R was tested with both single and double stranded substrates, no activity was found (Figure 2), similarly to what happened with RNase II_D209N mutant [28, 30]. Furthermore, we determined the exoribonucleolytic activity of wild-type RNase R and D280N mutant enzymes. Our results are consistent and confirm those obtained with the activity assays, being the activity of D280N mutant highly reduced when compared to the wild type enzyme (Table 3). To determine the involvement of this residue in RNA binding we calculated the dissociation constants of the wild type and mutant proteins by Surface Plasmon Resonance (SPR) using two different RNA substrates, a 25-mer and a poly(A) oligomers. We observed that the binding ability of the protein is not affected by the presence of this mutation, since D280N mutant presented a K_D value very similar to the wild type RNase R protein with both substrates tested: 2.2 ± 0.3 nM with 25-mer and 1.3 ± 0.2 nM with poly(A) for D280N *versus* 3.2 ± 0.4 nM with 25-mer and 1.2 ± 0.1 nM with poly(A) for RNase R (Table 4). These results confirm that, similarly to what happens with RNase II, this aspartate is indeed essential for the activity of RNase R without affecting RNA binding.

In RNase R, the four aspartates in the active center are located in the positions 272, 278, 280 and 281 and, like Asp-209 in RNase II, the equivalent Asp-280 in RNase R may well be the

only critical aspartate in this protein. Recent studies have shown that a mutation in aspartate 278 impairs the RNase R activity but the D278N mutant still retains 4% of activity [39]. Moreover, we can say that this residue plays a crucial role in the activity of all the proteins of this family since a mutation in the equivalent residue in RNase R of *Legionella pneumophila*, Asp-283, also led to the loss the exoribonucleolytic activity of the enzyme[40].

Tyr-324 is responsible for setting the final end-product in RNase R. In RNase II, the RNA molecule is stacked and clamped between two aromatic residues, Tyr-253 and Phe-358 [26] and it was previously shown that Tyr-253 is important for setting the final end-product of 4nts. In the Y253A mutant, the final product released was a 10nts fragment, which is the minimum length necessary for the RNA molecules to establish contacts between the catalytic and the anchoring regions, simultaneously [28]. In RNase R, the equivalent tyrosine is in position 324 (Supplementary Figure 1) and, in this work we wanted to see if this residue also played such an important role in the mechanism of RNA degradation. For that purpose, we introduced the point mutation Tyr324Ala into the pABA-RNR [15], and purified the respective protein. The exoribonucleolytic activity was analyzed with different RNA substrates and compared to the wild type. As shown in figure 2a, the mutant Y324A is capable of degrading the poly(A) substrate rendering a final product of 5nts instead of the usual 2nts observed for the wild-type. The same behavior is observed when we used a double stranded 16-30 substrate (Figure 2b). After this last cleavage event, the 5nts resultant fragment is no longer capable to establish the necessary contacts with the protein in order to achieve the cleavage of the substrate and is released. As such and similarly to what was shown with the equivalent residue in RNase II, Y253A [28], the corresponding residue in RNase R, Tyr-324, is also the one responsible for the establishment of the final end-product of this protein. When we measured the activity of Y324A, we observed that it retains only 2.5% of activity when compared with the wild type ($3.3 \pm 0.2 \text{ pmol.nmol}^{-1}.\text{min}^{-1}$ and $130.8 \pm 6.3 \text{ pmol.nmol}^{-1}.\text{min}^{-1}$, respectively) (Table 3), while in RNase II the equivalent mutation retained approximately 25% of activity [28]. However, when we compared the dissociation constants (K_D values) obtained by SPR, the mutant presented a ~6-fold reduction for both substrates tested (Table 4), a similar result to the one obtained for the Y253A mutant in RNase II [28]. These results suggest that the tyrosine residue in RNase R is also important for the binding of the RNA molecule at the 3'-end, but has a much more important function in the activity of the enzyme than in RNase II. This can be due to the fact that the second residue involved in the RNA clamping in RNase II, Phe-358 has no equivalent in RNase R, which can confer to Tyr-324 a more important function in RNA clamping.

The RNB domain of RNase II has catalytic activity and is able to bind to RNA. When the *E. coli* RNase II structure was solved it was possible to see that it is constituted by four domains instead of the three initially proposed. This protein is formed by two cold shock domains at the N-terminal region, a central catalytic domain RNB and a S1 domain at the C-terminal region [26]. Before the RNase II structure was known, previous studies were performed with truncated RNase II proteins, where the ΔCSD and RNB mutants included the regions that corresponded to the CSD2 [14]. In this work we constructed the genuine $\Delta\text{CSD1+2}$ and RNB mutants lacking both CSD1/CSD2 and CSD1/CSD2/S1 domains, respectively (Figure 1). We then induced and purified the truncated RNase II proteins and performed activity assays using different substrates.

When we analyzed the two truncated RNase II proteins regarding their ability to degrade the single stranded substrate poly(A) we observed that, like RNase II wild type, both RNase II $\Delta\text{CSD1+2}$ and RNase II_RNB were able to degrade the substrate until it reaches 4nts as a final-end product (Figure 3a). However, the activity of these truncated RNase II proteins is

very reduced when compared with the wild type enzyme, with RNase II_ΔCSD1+2 retaining only 0.03% of the enzyme's activity and RNase II_RNB less than 0.01% (Table 5). Taking into account that the two CSDs and S1 are the domains responsible for the RNA binding [14, 26], we can clearly say that the loss of activity observed in these mutants is caused by the reduction of their ability to bind to the RNA molecule. The determination of the K_D values showed that these mutants are able to bind to RNA molecules less efficiently than the wt enzyme for both substrates tested. While RNase II presents a K_D value of 6.5 ± 0.4 nM and 1.3 ± 0.4 nM for 25-mer and poly(A) substrates, respectively [29], the absence of both CSDs causes an increase of 4-fold in the K_D value, with RNase II_ΔCSD1+2 presenting K_D values of 24.1 ± 2.9 nM and 5.2 ± 0.6 nM for the 25-mer and poly(A) substrates, correspondingly (Table 6). The absence of all the RNA binding domains of the protein is responsible for a more pronounced reduction in RNA affinity, with the RNase II_RNB protein presenting almost a 10-fold reduction in the K_D values when compared to the wild type enzyme (Table 6).

When we analyzed the activity using a double stranded substrate, the 16-30ds, we verified that RNase II_ΔCSD1+2 is able to degrade a few more nucleotides than wt enzyme, releasing fragments with 20-22nts versus the 23-25nts released by RNase II (Figure 3b). This suggests that this mutant can go closer to the double stranded region maybe because the absence of both domains results in less steric hindrance, and allows the better entrance of double stranded molecules into the catalytic cavity. A similar result was obtained with the mutant lacking only the CSD1 [14]. However, when we tested the same substrate with the RNase II_RNB mutant, we observed that it was capable of degrading all the single stranded 3'-overhang of the substrate. The reaction stopped when RNase II_RNB mutant reached the single strand/double strand junction, and released a 16nts blunt-ended fragment (Figure 3b). It is possible to see that the band that corresponds to the 16nts fragment is slightly present in all reactions, even in the control. However, since the intensity of the bands is clearly more pronounced in the RNase II_RNB mutant, and no other products are observed, this indicates that the substrate is being degraded. As such, we can say that the 16nts is the final product that is being released by the mutant. Previous studies have shown that wild type RNase II has more difficulty in degrading the nucleotides adjacent to the double stranded region [39]. Our results confirm that the RNA-binding channel formed by the CSDs and S1 domains in RNase II could be the initial barrier for the degradation of double stranded substrates [14]. In fact, removal of the RNA-binding domains does allow RNase II to proceed further and the RNase II_RNB mutant protein is able to degrade all the way up to double stranded region.

The cleavage of double stranded RNA is a property of the RNB domain of RNase R.

The structural model of *E. coli* RNase R indicates that this protein shares a common 3D arrangement with *E. coli* RNase II and both enzymes have the same domain organization [28]. However, RNase R has an additional HTH (helix-turn-helix) domain at the N-terminal region and a basic region after the S1 domain (Figure 1). It is already known that the CSDs and S1 domains of RNase II are involved in RNA binding and that RNB domain is responsible for the catalytic activity of the enzyme [14]. This modular domain organization has also been postulated for RNase R, and the domains predicted to have the same function [28]. In order to confirm this hypothesis, we constructed truncated RNase R proteins which lack both CSDs and HTH and/or S1 domains as described in figure 1.

In order to see the contribution of each domain to the activity and binding of the enzyme, we performed activity assays with a 35-mer poly(A) substrate, calculated the exoribonucleolytic activity and determined the K_D values.

RNase R is able to degrade single-stranded substrates releasing as final product of the reaction a 2nts fragment [15] (Figure 4a). The same behaviour is observed for all the truncated

proteins, RNase R_ΔCSD1+2, RNase R_ΔS1 and RNase R_RNB (Figure 4a). However, when we compared the activity of the different enzymes with the wild type, it was possible to verify that all the truncated proteins analysed presented a significant reduction in their activity (Table 7). The absence of both cold shock domains in RNase R_ΔCSD1+2 protein causes a decrease of the activity of the enzyme in more than 1000-fold, from $130.8 \pm 6.3 \text{ pmol.min}^{-1}.\text{nmol}^{-1}$ to $0.2 \pm 0.04 \text{ pmol.min}^{-1}.\text{nmol}^{-1}$. This reduction is more pronounced when the S1 domain is absent, with RNase R_ΔS1 presenting an activity of $0.02 \pm 0.002 \text{ pmol.min}^{-1}.\text{nmol}^{-1}$. When only the RNB domain is present, the activity of the enzyme decreases more than 10.000-fold, to values lower than $0.01 \text{ pmol.min}^{-1}.\text{nmol}^{-1}$. These results show that both CSD and S1 domains are important for the activity of the enzyme, with S1 domain having a more important role.

Since it is postulated that CSDs and S1 domains are involved in RNA binding, the reduction in the activity observed in the truncated proteins can be due to a decrease in RNA binding ability. To confirm this hypothesis, we determined the K_D values of each protein by SPR using two different substrates. In fact, all the truncated protein showed a reduction in their K_D values when compared to the wild type enzyme for both substrates tested (Table 8). By analyzing the K_D values obtained for the truncated proteins, it is possible to see that the CSDs domains are more important for the RNA binding when compared with the S1 domain. This is illustrated by the fact that the RNase R_ΔCSD1+2 protein presented a 3-fold reduction in its binding ability, while the absence of S1 domain was responsible for only a 2-fold reduction (Table 8). When the three domains are absent, the reduction in the ability to bind RNA molecules is much more significant, with RNase R_RNB protein presenting a K_D value of $20.7 \pm 2.8 \text{ nM}$ for the 25-mer substrate and $7.4 \pm 0.4 \text{ nM}$ for the poly(A) substrate, values that are approximately 6-fold higher than those described for RNase R (Table 8). In RNase R_ΔS1 protein, the reduction in the activity is more pronounced than the verified for the RNase R_ΔCSD1+2 (Table 7), while the dissociation constants obtained for both substrates tested are just 2-fold higher for this protein when compared to wild type *versus* 3-fold higher for the RNase R_ΔCSD1+2 protein. This result led us to conclude that the CSDs and the S1 domains have different roles in RNA binding. The CSDs must be more important for the recruitment of the substrate, while S1 might help in the orientation and stabilization of the substrate in the catalytic cavity. It is interesting that the RNB domain, *per se*, is able to bind to RNA, although with a reduction of 6-fold when compared to the wild type. This also helps to explain the residual activity found in the RNase R_RNB protein.

As referred above, RNase R is able to degrade the double stranded substrate 16-30ds releasing a final product of 2nts (Figure 4b) [15]. When the truncated RNase R proteins were analysed with the same substrate, we verified that all of them were able to degrade it, releasing the characteristic 2nts fragment (Figure 4b).

RNase R RNA-binding domains discriminate which RNA molecules will be targeted for degradation. It has been suggested that RNase R needs a 3'- single-stranded overhang at least five nucleotides in length in order to degrade structured RNA molecules[41]. Then cleavage occurs at the single stranded 3' overhang and proceeds processively in the 5' direction, degrading the secondary structure [41, 42]. To verify which domains are responsible for the 3'-overhang requirement in RNase R we performed activity assays with the truncated RNase R proteins using a 16-16ds substrate (which lacks a 3'-overhang). As expected, wild type RNase R is not able to degrade the blunt-ended substrate that was tested (Figure 4c). Previous reports have also shown that the enzyme is not capable of degrading other blunt-ended substrates, even using more extreme reaction conditions (*i.e.* higher concentrations of enzyme and longer incubation periods) [41]. Surprisingly, the absence of CSDs and/or S1 domains resulted in the ability to degrade the 16-16ds substrate as shown in figure 4c. These results

show that the binding domains (CSD1, CSD2 and S1) are blocking the entrance of the double stranded substrates into the catalytic channel. In their absence, the substrate is free to enter into the catalytic cavity and cleavage can occur.

Together, these results show that the RNB domain of RNase R is sufficient for the activity of the enzyme. This domain is also capable of binding to RNA substrates, although with less efficiency, and this explains its reduced activity. The decrease in RNA affinity upon deletion of either the CSDs or the S1 domain confirms that these regions indeed function as RNA-binding domains. They are also responsible for the selective degradation of double stranded substrates that contain a 3' single stranded overhang of five or more nucleotides. In fact, both in prokaryotes and eukaryotes, the RNAs targeted for degradation usually contain a short poly(A) tail [43]. In case of RNase R, the presence of the binding domains is important for the degradation of only structured RNA molecules that have an overhang of, at least, 5nts length. This suggests that this protein is capable of degrading RNA molecules only when they are targeted to that purpose. As such, the discrimination of which molecules are targeted for decay is made by the CSD1, CSD2 and S1 domains.

In conclusion, the results presented in this study gave an important breakthrough in the understanding of the RNase R mechanism of action. We have identified the Tyr-324 residue that is responsible for setting the final end-product releasing 5nts instead of the usual 2nts observed for the wild-type RNase R. This residue is fully conserved in all domains of life and was proved to have the same function in RNase II. We also show that the Asp-280 residue, present in the RNase R active site, is crucial for the activity of the enzyme but not for RNA binding.

We have demonstrated that RNB domains of RNase II and RNase R are able to degrade RNA, even in the absence of the RNA binding domains. The most striking result is that, contrary to the existing literature, the RNB domain of RNase R is sufficient for the degradation of double stranded substrates even in the absence of a 3'-overhang. The RNA binding domains present in the wild type RNase R prevent the degradation of blunt dsRNA molecules. The discrimination of which molecules will be susceptible to degradation by RNase R is made by the CSD1, CSD2 and S1 domains that target the degradation of RNAs that are "tagged" by a 3'-tail. These results can have important implications for the study of poly(A)-dependent degradation mechanisms.

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Tables

Table 1: Plasmids used in this study.

Plasmid	Relevant characteristic	Reference
pFCT6.1	gene <i>rnb</i> cloned into pET15b, AmpR	Cairrão <i>et al.</i> , 2001
pABA-RNR	gene <i>rnr</i> cloned into pET15b, AmpR	Amblar <i>et al.</i> , 2007
pABA-RNR_D280N	Expresses RNase R where the Asp280 was changed by an Asn	This work
pABA-RNR_Y324A	Expresses RNase R where the Tyr324 was changed by an Ala	This work
pFCT_XhoI521	pFCT6.1 with a <i>XhoI</i> restriction site in position 521 of <i>rnb</i> gene	This work
pABA-RNR_NdeI549	pABA-RNR with a <i>NdeI</i> restriction site in position 549 of <i>rnr</i> gene	This work
pFCT_ΔCSDs	Expresses RNase II lacking CSD1 and CSD2 domains	This work
pFCT_RNB	Expresses RNase II lacking CSD1, CSD2 and S1 domains	This work
pABA-RNR_ΔCSDs	Expresses RNase R lacking HTH, CSD1 and CSD2 domains	This work
pABA-RNR_ΔS1	Expresses RNase R lacking S1 domain	This work
pABA-RNR_RNB	Expresses RNase R lacking HTH, CSD1, CSD2 and S1 domains	This work

Table 2: Primers used in this study.

Primer	Sequence (5' → 3') ^{ab}	Purpose
Asp280Asn_Fw	CGCCCGGGACTTTAACGATGC	Introduces D280N mutation into pABA-RNR
Asp280Asn_Rev	GCATCGTTAAAGTCCCGGGCG	Introduces D280N mutation into pABA-RNR
Tyr324Ala_Fw	CCGTGGGACGTCGGTGGCCTTCCCTTCGC	Introduces Y324A mutation into pABA-RNR
Tyr324Ala_Rev	GCGAAGGGAAGGCCACCGACGTCCACGG	Introduces Y324A mutation into pABA-RNR
pFCT_XhoI512_Fw	CATCACTTTTGGTCTCGAGCACTTTGTACCGT	Introduces <i>XhoI</i> restriction site into pFCT6.1 at the position 512
pFCT_XhoI521_Rev	ACGGTACAAAGTGCTCGAGACCAAAGTGATG	Introduces <i>XhoI</i> restriction site into pFCT6.1 at the position 512
RNB_Stop1735_Fw	GCCGGGTAAGACACGCGTTTCGC	Introduces stop codon into pFCT6.1 at the position 1735
RNB_Stop1735_Rev	GCGAAACGCGTGTCTTACCCGGC	Introduces stop codon into pFCT6.1 at the position 1735
RNR_NdeI549_Fw	GTCGAAGTGCTGGGCGACCATATGGGCACC	Introduces <i>NdeI</i> restriction site into pABA-RNR at the position 549
RNR_NdeI549_Rev	GGTGCCCATATGGTCCGCCCAGCACTTCGAC	Introduces <i>NdeI</i> restriction site into pABA-RNR at the position 549
RNR_Stop1927_Fw	GTGTGACTTCATGCTCGACCAGTAAGG	Introduces stop codon into pABA-RNR at the position 1927
RNR_Stop1927_Rev	CCTTACTGGTTCGAGCATGAAGTCACAC	Introduces stop codon into pABA-RNR at the position 1927

a) Bases underlined indicate restriction endonuclease sites

b) Bases in bold indicate aminoacid changes

Table 3: Exoribonucleolytic activity of wild-type RNase R and point mutant enzymes.

Exoribonucleolytic activity was assayed using a 35nt poly(A) chain as substrate. Activity assays were performed in triplicate as described in "Experimental".

Protein	Protein Activity ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{nmol}^{-1}$)	Relative Activity (%)
wt RNase R	130.8 \pm 6.3	100
D280N	<<0.01	<<0.01
Y324A	3.3 \pm 0.2	2.5

Table 4: RNA binding affinity of wild-type RNase R and point mutant enzymes.

The dissociation constants (K_D) were determined by Surface Plasmon Resonance using Biacore 2000 with a 25-nt RNA oligomer (5'-Biotin-CCC GAC ACC AAC CAC UAA AAA AAA A-3') and 30nts poly(A) as substrates as described in "Experimental".

Protein	K_D 25-mer	Relative K_D 25-mer	K_D poly(A)	Relative K_D poly(A)
wt RNase R	3.2 \pm 0.4	1.0	1.2 \pm 0.1	1.0
D280N	2.2 \pm 0.3	0.7	1.3 \pm 0.2	1.1
Y324A	20.4 \pm 1.4	6.4	6.7 \pm 0.3	5.6

Table 5: Exoribonucleolytic activity of wild-type and truncated RNase II proteins.

Exoribonucleolytic activity was assayed using a 35nt poly(A) chain as substrate. Activity assays were performed in triplicate as described in "Experimental".

Protein	Protein Activity ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{nmol}^{-1}$)	Relative Activity (%)
wt RNase II	299.4 \pm 36.0	100
RNase II_ΔCSD	0.1 \pm 0.01	0.03
RNase II_RNB	<<0.01	<<0.01

Table 6: RNA binding affinity of wild-type and truncated RNase II proteins.

The dissociation constants (K_D) were determined by Surface Plasmon Resonance using Biacore 2000 with a 25-nt RNA oligomer (5'-Biotin-CCC GAC ACC AAC CAC UAA AAA AAA A-3') and 30nts poly(A) as substrates as described in "Experimental".

Protein	K_D 25-mer	Relative K_D 25-mer	K_D poly(A)	Relative K_D poly(A)
wt RNase II	6.5 \pm 0.4	1.0	1.3 \pm 0.4	1.0
RNase II_ΔCSD	24.1 \pm 2.9	3.7	5.2 \pm 0.6	4.0
RNase II_RNB	59.9 \pm 6.0	9.2	11.7 \pm 2.1	9.0

Table 7: Exoribonucleolytic of wild-type and truncated RNase R proteins.

Exoribonucleolytic activity was assayed using a 35nt poly(A) chain as substrate. Activity assays were performed in triplicate as described in "Experimental".

Protein	Protein Activity ($\text{pmol}\cdot\text{min}^{-1}\cdot\text{nmol}^{-1}$)	Relative Activity (%)
wt RNase R	130.8 ± 6.3	100
RNase R_ΔCSD	0.2 ± 0.04	0.1
RNase R_ΔS1	0.02 ± 0.002	0.02
RNase R_RNB	$\ll 0.01$	$\ll 0.01$

Table 8: RNA binding affinity of wild-type and truncated RNase R proteins.

The dissociation constants (K_D) were determined by Surface Plasmon Resonance using Biacore 2000 with a 25-nt RNA oligomer (5'-Biotin-CCC GAC ACC AAC CAC UAA AAA AAA A-3') and 30nts poly(A) as substrates as described in "Experimental".

Protein	K_D 25-mer	Relative K_D 25-mer	K_D poly(A)	Relative K_D poly(A)
wt RNase R	3.2 ± 0.4	1.0	1.2 ± 0.1	1.0
RNase R_ΔCSD	9.6 ± 1.4	3.0	3.1 ± 0.1	2.6
RNase R_ΔS1	5.4 ± 0.3	1.7	2.1 ± 0.2	1.8
RNase R_RNB	20.7 ± 2.8	6.5	7.4 ± 0.4	6.2

Figure Legends

Figure 1. Linear representation of the domains of wild type RNase II, RNase R and its derivatives proteins.

Figure 2. Exoribonuclease activity of RNase R wild-type and point mutants enzymes.

Activity assays were performed as described under “Experimental” using a poly(A) chain of 35 nt (A) or a 30-mer oligoribonucleotide hybridized to the complementary 16mer oligodeoxyribonucleotide, thus obtaining the corresponding double stranded substrate 16-30ds (B). The proteins used and their respective concentrations are shown. The wild-type enzyme was used as control. Samples were taken during the reaction at the time points indicated and reaction products were analyzed in a 20% polyacrylamide/7M urea gel. Control reactions with no enzyme added (Ctrl) were incubated at the maximum reaction time for each protein. Length of substrates and degradation products are indicated in the figure.

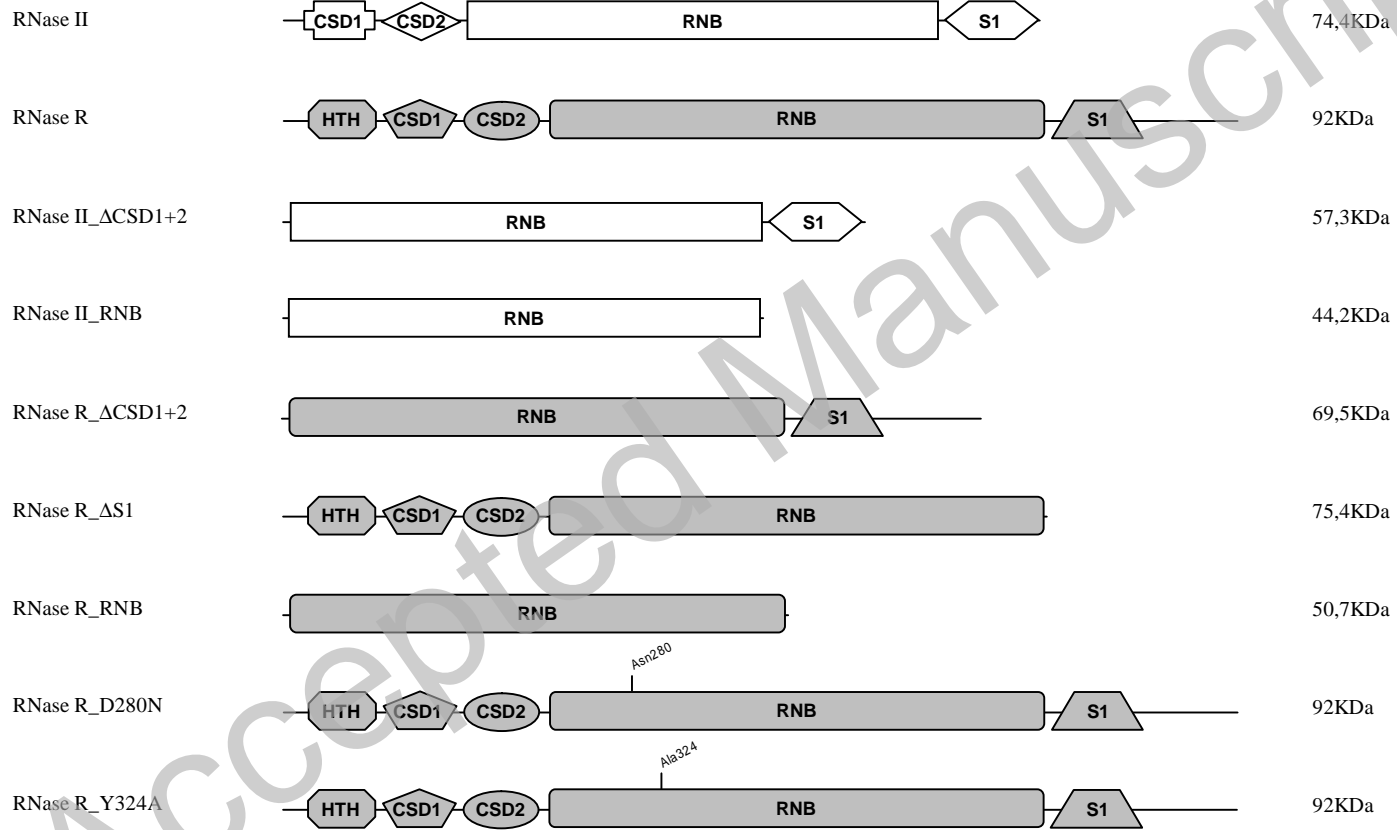
Figure 3. Exoribonuclease activity of wild-type and truncated RNase II enzymes.

Activity assays were performed as described under “Experimental” using a poly(A) chain of 35 nt (a) or a 30-mer oligoribonucleotide hybridized to the complementary 16mer oligodeoxyribonucleotide, thus obtaining the corresponding double stranded substrate 16-30ds (b). The proteins used and their respective concentrations are shown. The wild-type enzyme was used as control. Samples were taken during the reaction at the time points indicated and reaction products were analyzed in a 20% polyacrylamide/7M urea gel. Control reactions with no enzyme added (Ctrl) were incubated at the maximum reaction time for each protein. Length of substrates and degradation products are indicated in the figure.

Figure 4. Exoribonuclease activity of wild-type and truncated RNase R enzymes.

Activity assays were performed as described under “Experimental” using a poly(A) chain of 35 nt (a), a 30-mer oligoribonucleotide hybridized to the complementary 16mer oligodeoxyribonucleotide, thus obtaining the corresponding double stranded substrate 16-30ds (b) or a 16-mer oligoribonucleotide hybridized to the complementary 16mer oligodeoxyribonucleotide, thus obtaining the corresponding double stranded substrate 16-16ds. The proteins used and their respective concentrations are shown. The wild-type enzyme was used as control. Samples were taken during the reaction at the time points indicated and reaction products were analyzed in a 20% polyacrylamide/7M urea gel. Control reactions with no enzyme added (Ctrl) were incubated at the maximum reaction time for each protein. Length of substrates and degradation products are indicated in the figure.

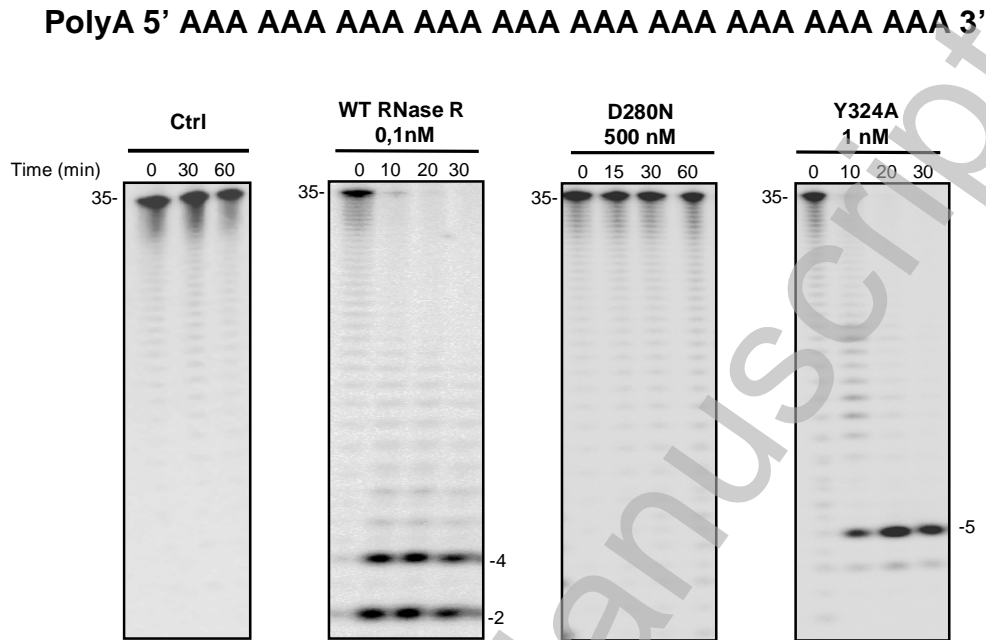
Figure 1



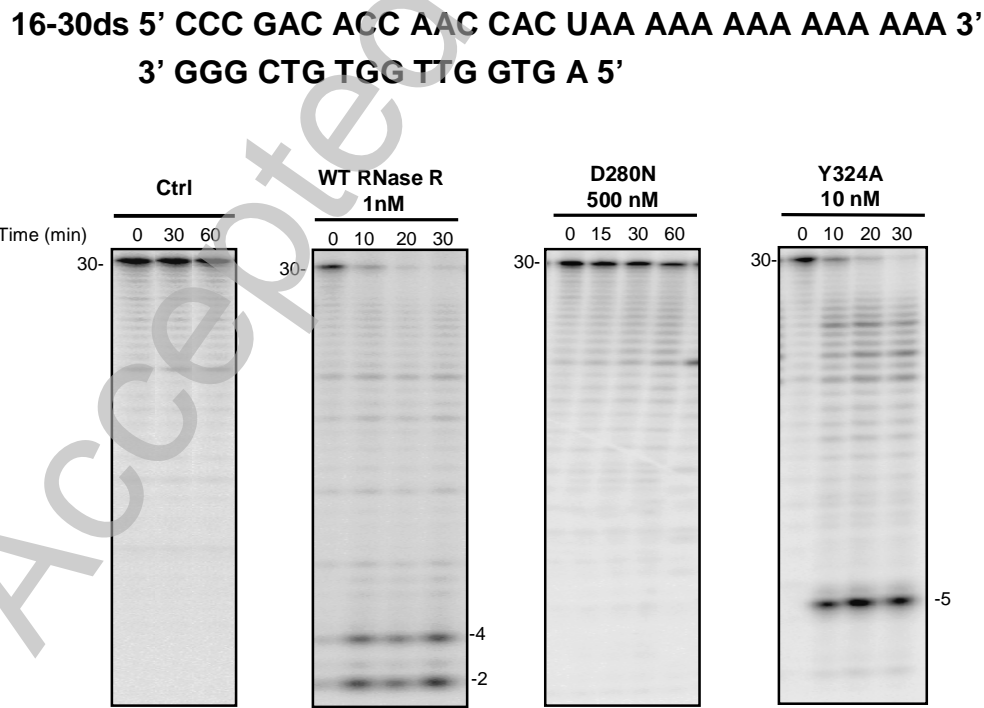
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Figure 2

a



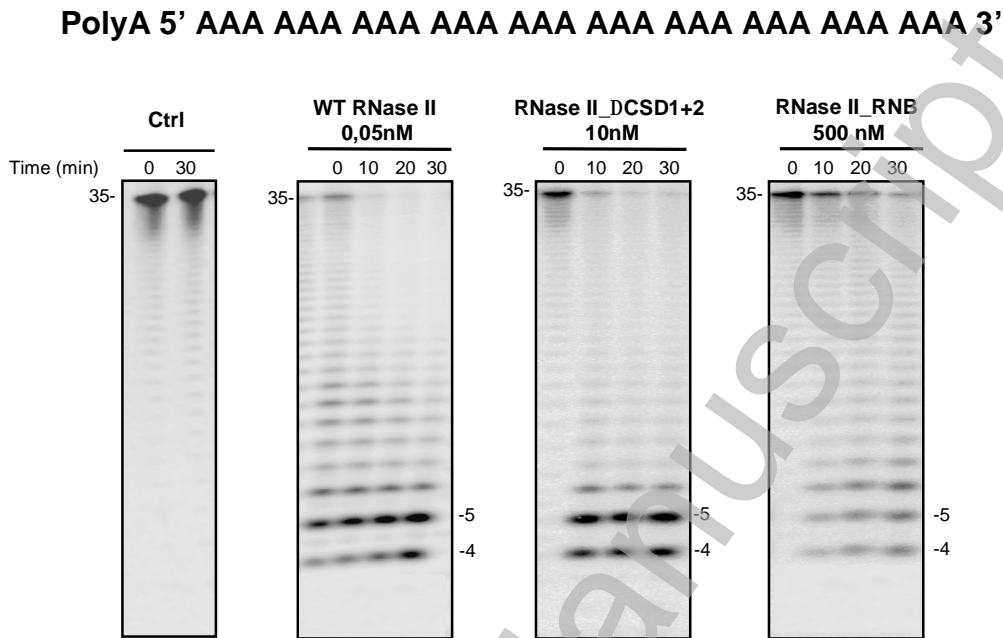
b



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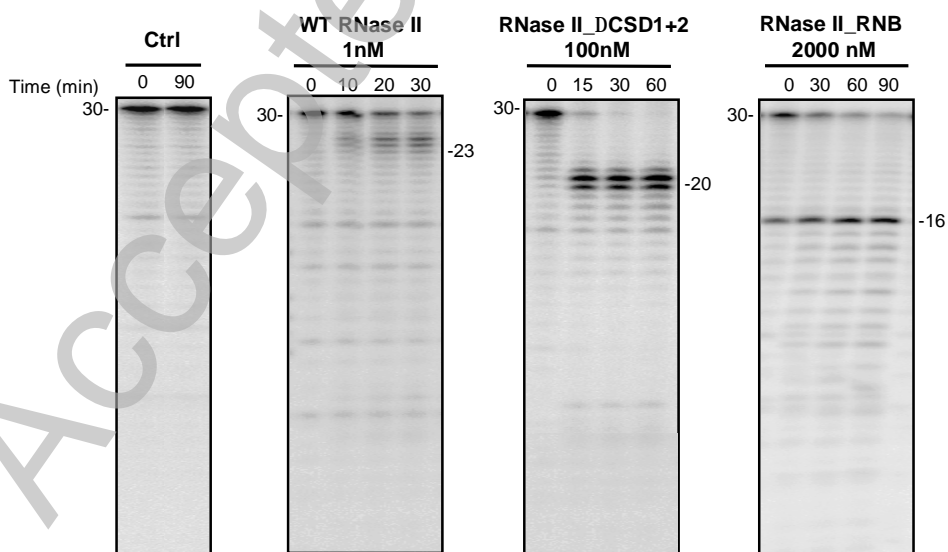
Figure 3

a



b

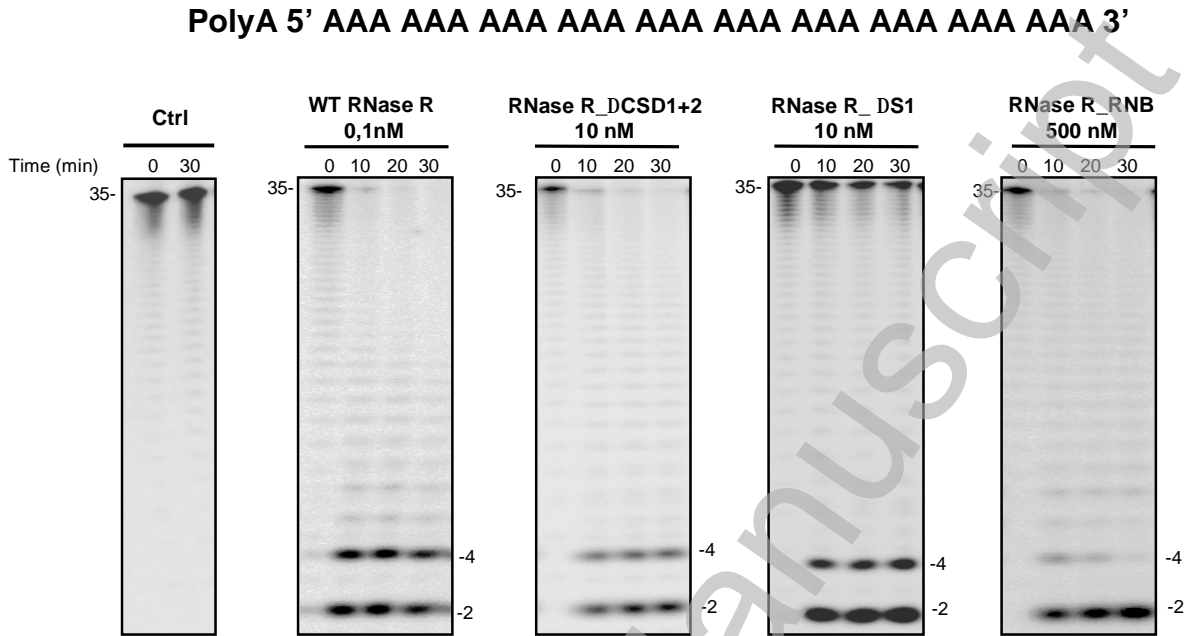
16-30ds 5' CCC GAC ACC AAC CAC UAA AAA AAA AAA AAA 3'
3' GGG CTG TGG TTG GTG A 5'



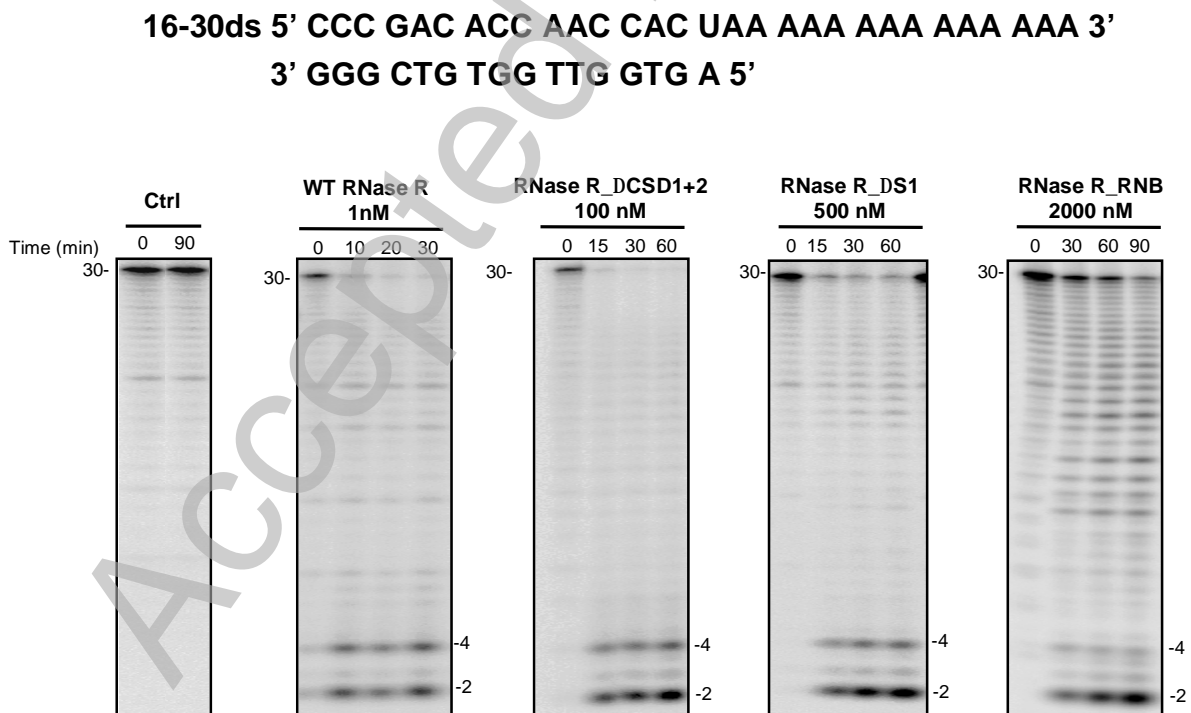
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Figure 4

a



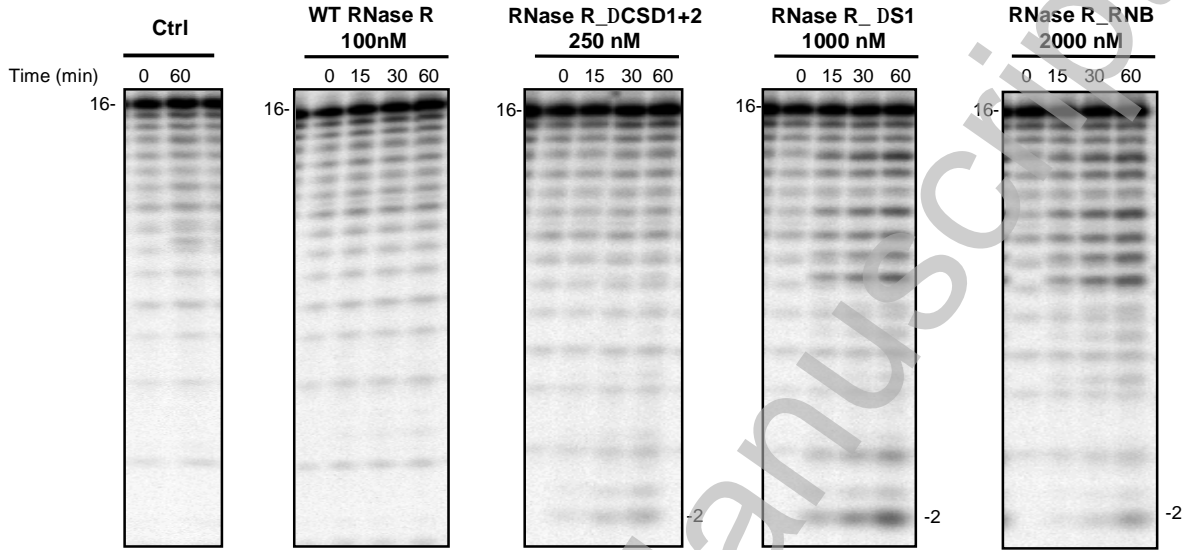
b



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c

16-16ds 5' CCC GAC ACC AAC CAC U 3'
3' GGG CTG TGG TTG GTG A 5'



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