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The sequence of sites recognised by a member of the RNase E/G family can control the maximal rate of cleavage, while a 5′-monophosphorylated end appears to function cooperatively in mediating RNA binding

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ABSTRACT

Members of the RNase E/G family are multimeric, 5′-end-sensing, single-strand-specific endoribonucleases that are found in chloroplasts as well as bacteria, and have central roles in RNA processing and degradation. A well-studied member of this family is *Escherichia coli* RNase G. Recently, we have shown that the interaction of this enzyme with a 5′-monophosphorylated end can enhance substrate binding *in vitro* and the decay of mRNA *in vivo*. We show here that a single-stranded site despite not being sufficient for rapid cleavage makes a substantial contribution to the binding of RNase G. Moreover, we find that the sequence of a site bound by RNase G can moderate the maximal rate by at least an order of magnitude. This supports a model for the RNase E/G family in which a single-stranded segment(s) can cooperate in the binding of enzyme that subsequently cleaves preferentially at another site. We also provide evidence that in order to promote cleavage a 5′-monophosphorylated end needs to be linked physically to a single-stranded site, indicating that it functions cooperatively. Our results are discussed in terms of recent X-ray crystal structures and models for the initiation of bacterial mRNA degradation.

Introduction

Two paralogous endoribonucleases, RNase E and RNase G, are involved in both the processing and degradation of RNA in *Escherichia coli* [1]. Studies of *E. coli* RNase E have revealed that it cleaves within single-stranded segments that tend to be rich in A and/or U nucleotides [2]. Although nucleotides at certain positions have been identified that can affect the rate of cleavage [3], the range of sequences cleaved by RNase E is broad [2,4–9] and includes homopolymeric runs of A or Us [3,10,11]. The sequence requirements for cleavage by RNase G have not been investigated, but its specificity overlaps that of RNase E [12,13]. For example, RNase G cuts a model oligonucleotide substrate derived from the major site of RNase E cleavage at the 5′ end of RNAI, the antisense RNA regulator of ColE1-type plasmid replication [14], at the same position as its paralogue [15,16].

Members of the RNase E/G family have been found in chloroplast and in many, but not all, divisions of bacteria [17,18]. In *E. coli*, RNase G through almost its entire length is similar in sequence to the N-terminal catalytic half of RNase E [19,20]. Additional domains that are not required for catalysis are present in RNase E of *E. coli* and other bacteria [17]. For both *E. coli* RNase E and G, the fastest rate of cleavage of several substrates has been found to be dependent on the presence of a 5′ end that is both monophosphorylated and unpaired [15,21,22]. Recently, *E. coli* has been shown to have a pyrophosphatase that can convert the triphosphate at the 5′ end of nascent transcripts to a monophosphate [23]. Inactivation of this enzyme appears to result in the stabilisation of many, but not most of the transcripts in *E. coli* [24]. This indicates that while 5′-monophosphate-dependent pathways make a significant contribution to mRNA degradation other pathways exist in *E. coli*. A pocket that can bind a monophosphate group and the first nucleotides at a 5′ end has been revealed in crystal structures of the N-terminal half of RNase E [25]. This pocket, which is associated with a subdomain called the 5′ sensor, does not overlap with the site of catalysis, which is at the other end of an RNA-binding channel that can accommodate a single-stranded (ss) segment (Fig. 1S). The channel is formed by a conformational change that brings together an S1- and a DNase I-like subdomain [26]. The latter subdomain contains residues that coordinate a magnesium ion implicated in catalysis. Residues of *E. coli* RNase E that can contact a 5′-monophosphorylated end, form the ssRNA-binding channel and coordinate the catalytic magnesium ion are conserved in RNase G [20,25]. This has allowed structure-based studies of the function of RNase G as well as RNase E [16,25,27,28].

Escherichia coli RNase E forms a tetramer [25,29], which is better described as a dimer of a principal dimer in which residues from different protomers contribute to each of two identical ssRNA-binding channels and active sites (Fig. 1S). RNase G lacks segments that ap-

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pear to stabilise the dimer: dimer interface in *E. coli* RNase E and is purified predominantly as a dimer [16,30]. The finding that RNase E and RNase G are multimeric raises the possibility of cooperative binding. Recently, we have shown for *E. coli* RNase E that the joining of single-stranded oligonucleotides to form a multimeric substrate can facilitate rapid cleavage in the absence of an unpaired 5′-monophosphorylated end *in vitro* [28]. We also found that rapid cleavage of *cspA* mRNA by RNase E, which occurs predominantly at a single site in the 3′-untranslated region (UTR) [31], is not dependent on interaction with a 5′-monophosphorylated end and that several single-stranded sites in addition to the major site of cleavage are recognisable by RNase E [28]. The above led us to propose that one or more of the additional sites in *cspA* mRNA functioned cooperatively with the site in the 3′ UTR to facilitate its cleavage. A key facet of this model is that RNase E (and presumably other members of its family) can preferentially cleave one of the single-stranded segments with which it interacts simultaneously. Here we show using *E. coli* RNase G as a model that the sequence of a single-stranded segment that is bound can moderate by an order of magnitude its maximal rate of cleavage. RNase G was chosen, as unlike its paralogue, we are able to assay its binding of RNA quantitatively using fluorescence anisotropy [16]. The system for analysing the contribution of sequence was simplified by using substrates with one single-stranded segment. Cleavage of these substrates was facilitated by incorporating a 5′-monophosphate group. We also present the results of experiments that support our notion that a 5′-monophosphorylated end does not serve primarily as an allosteric activator of catalytic activity, but provides a foothold that can function cooperatively in substrate binding, as proposed for single-stranded segments that are not highly cleavable [28].

Materials and methods

Purification of RNase G. Recombinant wild-type enzyme and the D303N mutant were purified using a protocol that is based on immobilised metal affinity chromatography, and has been described previously [16]. Protein concentrations were established using a modified Bradford assay (BioRad) and SDS–PAGE.

Assay of RNA cleavage. The conditions were adapted from an earlier protocol [32], as described previously [16]. The oligonucleotide substrates were 3′ fluorescein-labelled versions of BR13 (5′-GGGACAGUAUUUG-3′) [33] and 9S13b (5′-GGCAUCAAAUAAA), a 13-mer version of 9Sb [34]. These substrates were synthesised and purified by Dharmacon (USA) and MWG (Germany), respectively. Prior to the start of a reaction, the components were pre-warmed at 37 °C for 20 min [28]. The reaction products were analysed by gel electrophoresis under denaturing conditions. Labelled species were detected using a Molecular Imager FX-Pro multi-imager system (BioRad) and quantified using AIDA software (Raytest Isotopenmessgeräte GmbH), as described previously [16].

Assay of RNA binding. Fluorescence anisotropy measurements were performed using a Spex® FluorLog® Tau spectrofluorometer (HORIBA Jobin Yvon) and the 3′ fluorescein-labelled substrates described in the previous section. Protein was added in storage buffer (20 mM Tris–HCl [pH 7.6], 500 mM NaCl, 10 mM MgCl$_2$, 10 mM DTT, 0.5 mM EDTA, and 5% [v/v] glycerol), since overnight dialysis of the proteins into our standard cleavage-assay buffer causes some precipitation [16]. Binding isotherms were fitted using least squares regression to an equation describing the equilibrium, $\mathrm{R} + \mathrm{E} \leftrightarrow \mathrm{RE}$ (R, RNA; E, enzyme; RE, RNA–enzyme complex), as described previously [16].

Results and discussion

Previous work has revealed that 9Sb, a decanucleotide substrate based on the sequence of the site required for the maturation of the 3′ end of 5S rRNA, is cleaved slowly by *E. coli* RNase E *in vitro* despite having a 5′-monophosphate [34]. This was associated with the lack of a G nucleotide at a central position [34], which has been shown in other sequence contexts to influence the rate of cleavage under non-saturating conditions [3,34] and is found in many, but not all, sites of RNase E cleavage in *E. coli* [4,8]. To investigate whether 9Sb is also cleaved slowly by RNase G, we used a derivative called 9S13b (5′-GGCAUCAAAUAAA) that has a GGC triplet at its 5′ end to make it the same length as BR13 (5′-GGGACAGUAUUUG-3′) [33], a model substrate that is based on the RNase E site at the 5′ end of RNAI [14,35] and has been used in previous studies of RNase G [15,16,28]. We found that 9S13b was cleaved ~80-fold slower than BR13 (Fig. 1). This is similar to what was found previously for RNase E [34]. RNase G cleaved 9S13b at multiple sites (Fig. 1) and these were mapped to the 3′ side of nucleotides 7–10 by running against size markers (data not shown). This pattern of cleavage can be explained in terms of what is known about RNase E. While RNase E can cut at numerous positions within AU-rich segments that lack a G determinant [2,3,11], it is restricted to sites that are more than 3 and 7 nt from the 3′ and 5′ end, respectively [3,11]. An explanation for these 'end effects' is provided by the crystal structure of the N-terminal half of RNase E: maximum contact with the ssRNA-binding channel appears to require 3 nt downstream of a sessile bond and the minimum distance between the sites of catalysis and 5′-monophosphate binding is estimated to be 7 nt [25].

Having identified 9S13b as an oligonucleotide substrate with a sequence that is cleaved slowly by RNase G, we assayed its binding using fluorescence anisotropy [36,37] and RNase G D303N [16], an active site mutant that lacks an aspartate involved in the coordina-

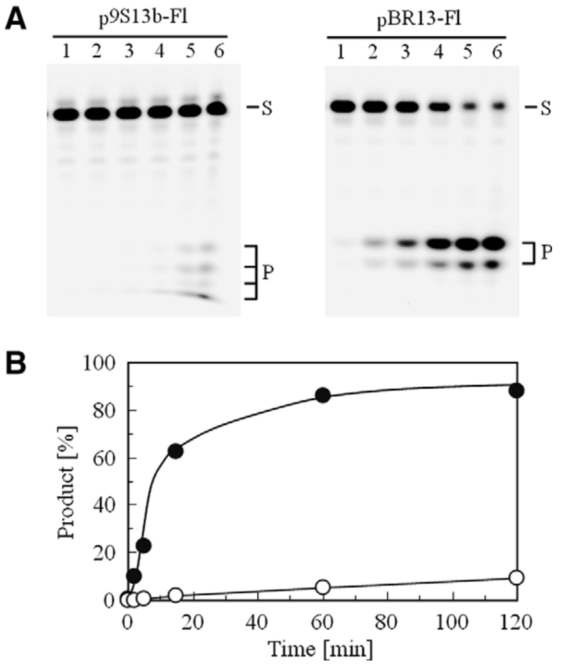

Fig. 1. Cleavage of 9S13b and BR13 by *E. coli* RNase G. (A) Analysis of reaction products by gel electrophoresis. The assays contained 5 and 250 mM of RNase G and 5′-monophosphorylated substrate, respectively. The identity of each substrate is shown at the top of the panel. Lanes 1–6 correspond to time points of 0, 2, 5, 15, 60 and 120 min, respectively. The positions of substrate (S) and products (P) are indicated on the right. The samples were run on a denaturing 15% polyacrylamide gel. (B) Quantification of the reaction products. The 3′-labelled species were detected using a Molecular Imager FX-Pro multi-imager system (BioRad) and quantified using AIDA software (Raytest Isotopenmessgeräte GmbH), as described previously [16]. Closed and open circles represent data-points for BR13 and 9S13b, respectively.

tion of magnesium [25]. The conditions were as described previously [16]. The addition of RNase G D303N to 9S13b caused an increase in anisotropy that began to plateau at higher concentrations (Fig. 2A). Binding to 9S13b was highly dependent on the presence of a 5′-monophosphate group, as found previously for BR13 [16]. By fitting the data to a 1:1 binding model, an apparent K_D of 495 ± 68 nM was obtained for the 5′-monophosphorylated RNA. This value was only 3-fold higher than the apparent K_D of 162 ± 33 nM obtained previously for BR13 under identical conditions [16], indicating that reduced binding affinity is insufficient to explain the ~80-fold slower rate of cleavage of 9S13b under non-saturating conditions. Thus, we undertook a Michaelis–Menten analysis of 9S13b. From an Eadie–Hofstee plot, we obtained values of K_M and k_{cat} of 670 ± 74 nM and 0.29 ± 0.02 min^{-1}, respectively (Fig. 2B). The values obtained previously for RNase G cleavage of BR13 were 120 ± 17 nM and 3.0 ± 0.1 min^{-1}, respectively [16]. The k_{cat}/K_M ratio for 9S13b is ~60-fold lower than for BR13, in agreement with its slower rate of cleavage under non-saturating conditions (Fig. 1). The finding that the k_{cat} for 9S13b is ~10-fold lower than BR13 indicates that the sequence of single-stranded segments recognised by RNase G can have a significant effect on the maximum rate of turnover in the active site. Experiments are underway to determine the sequence requirements for the binding of single-stranded segments by members of the RNase E/G family. As 9S13b is based on the sequence of a site required for rRNA processing in *E. coli*, we suspect that some sequences will be cleaved much slower, while being bound with an affinity similar to, or higher than BR13.

The finding that the sequence of a single-stranded region can also influence the affinity of the interaction with RNase G (the apparent K_D and K_M values for 9S13b were 3.0- and 5.6-fold higher, respectively, than those for BR13) prompted us to study whether interaction with a 5′-monophosphorylated end might be insufficient, despite being required, for high affinity binding to a substrate with only one single-stranded site. This possibility was investigated using a competition reaction. We assayed the ability of an unlabelled 5′-monophosphorylated guanosine triplet, which corresponds to the sequence at the 5′ end of BR13 and provides all of the contact points identified as being required for interaction with the 5′ end-binding pocket [25], to inhibit the cleavage of 5′-monophosphorylated BR13 by RNase G. We also assayed a 5′-monophosphorylated pentanucleotide (AUUUG), which also provides all of the contacts for the 5′ end-binding pocket or alternatively the ssRNA-binding channel (Fig. 1S) [25]. Incubation of both these fragment in 500-fold excess caused only a slight reduction in the rate of cleavage of 5′-labelled BR13 (Fig. 3). This was in contrast to incubation with an excess of unlabelled BR13, which as expected competed effectively and reduced substantially the rate of cleavage of the labelled substrate. The simplest interpretation of these results is that interaction with a single-stranded segment is an important determinant of the overall affinity of RNA binding, consistent with our finding that sequence can influence binding as well as cleavage by RNase G (Fig. 2).

Moreover, we found that a single-stranded region needs to be linked physically to a 5′-monophosphorylated-end segment in order for the latter to promote cleavage. Providing an excess of 5′-monophosphorylated GGG triplet failed to stimulate RNase G cleavage of 5′-hydroxylated BR10 (5′ ACAGUAUUUG) (Fig. 4), a decanucleotide derivative of BR13 [33]. In contrast, BR13 (which is the product of linking these two segments) was cleaved rapidly by RNase G under the same conditions. Similar results have been obtained for RNase E (data not shown). Thus, rapid cleavage of a single-stranded region appears to occur only as part of a multipartite interaction. This may provide a means of increasing the selectivity of an endonuclease with broad sequence specificity. Although an isolated 5′-monophosphorylated-end segment does not appear to serve as a potent allosteric activator of the cleavage of RNA (Fig. 4), we do not exclude the possibility that a 5′-monophosphorylated end as part of a substrate can contribute to induced-fit as well as initial binding. When associated with 5′-monophosphorylated RNA the conformation of RNase E has been found to be more compact [29].

In the crystal structure of RNase E complexed with 5′-monophosphorylated oligonucleotide substrates, there appears to be little sequence recognition within the ssRNA-binding channel [25]. However, a novel oligonucleotide-based assay has identified sequence determinants that influence the specificity and efficiency of RNase E cleavage [3]. These findings can be reconciled should the sequence determinants that promote specific cleavage do so by generating an RNA conformation that is favoured by RNase E [25]. However, to our knowledge, there are currently insufficient solution structures of single-stranded RNAs with variations in sequence to predict the effect of specific nucleotides on RNA conformation. Another possibility is that the S1 subdomain makes sequence-specific contacts with RNA when RNase E is in an open conformation [26], but these are replaced on closing of the RNase E structure and formation of the RNA-binding channel. S1 subdomains in other proteins are known to contribute to sequence-specific recognition [38].

Regardless of the details of sequence-specific recognition, the work reported here shows that the sequences of sites bound by

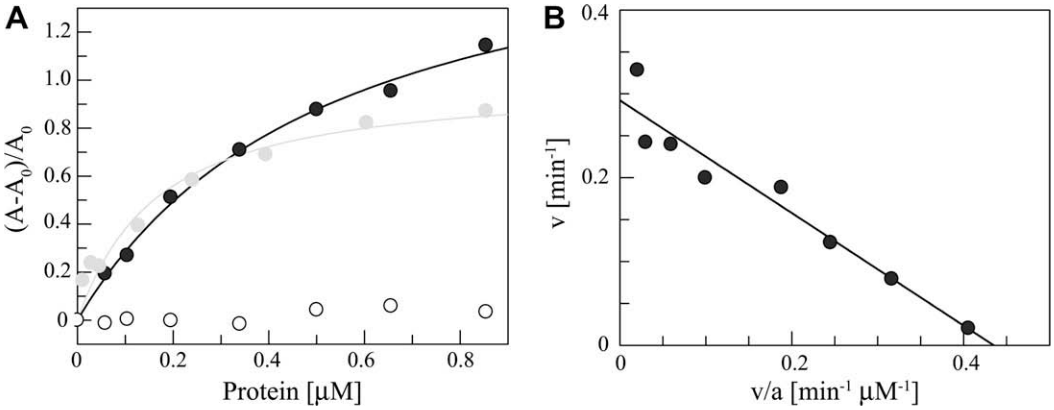

Fig. 2. RNA binding and Michaelis–Menten parameters. (A) The assay of RNA binding using fluorescence anisotropy. *A* is the anisotropy value measured at a given condition; A_0 is the anisotropy value before addition of protein. Black closed and open circles represent data-points for 9S13b with a 5′-monophosphate and hydroxyl, respectively. The nonlinear fit obtained for 5′-monophosphorylated 9S13b is shown as a black line. Data obtained under the same conditions for 5′-monophosphorylated BR13 [16] is shown in light grey. (B) Michaelis–Menten analysis of the cleavage of 5′-monophosphorylated 9S13b. The data is presented as an Eadie–Hofstee plot. The concentration of RNase G was 50 nM, while the concentration of substrate was varied between 50 nM and 16 μM. The negative slope and the *y*-intercept of this plot represent the K_M and k_{cat}, respectively.

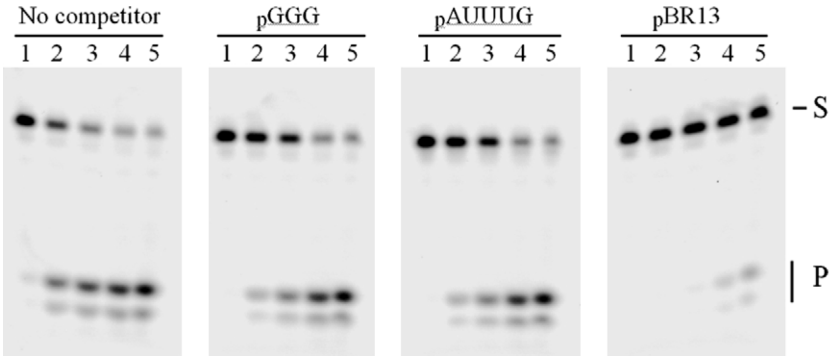

Fig. 3. Competition analysis of short, 5′-monophosphorylated fragments. RNase G was incubated with 5′-monophosphorylated, 3′-labelled BR13 in the absence or presence of unlabelled G triplet, a pentanucleotide (5′-AUUUG) or BR13. The concentrations of RNase G, 3′-labelled BR13 and each of the 5′-monophosphorylated, unlabelled RNA species were 10 nM, 75 nM and 37.5 μM, respectively. Lanes 1–5 correspond to time points of 0, 5, 15, 60 and 120 min, respectively. The labelling is as in Fig. 1.

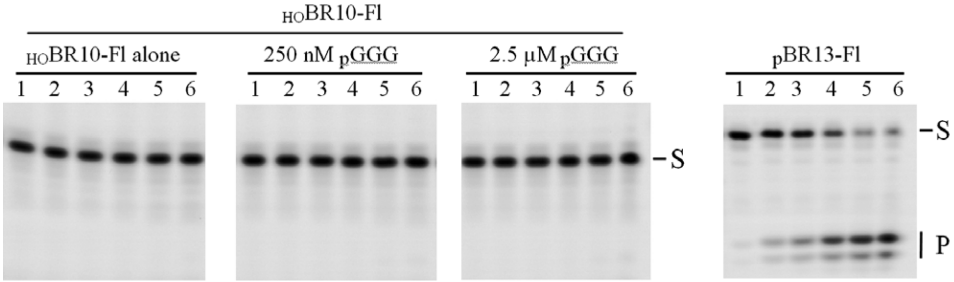

Fig. 4. Evidence for the 5′ end interaction functioning cooperatively in RNA binding. RNase G was incubated with 5′-hydroxylated, 3′-labelled BR10 ($_{OH}$BR10-Fl) in the absence and presence of 5′-monophosphorylated, unlabelled GGG ($_{p}$GGG). As a control, RNase G was also incubated with 5′-monophosphorylated BR13 ($_{p}$BR13-Fl). The concentrations of enzyme and labelled substrate were 5 and 250 nM, respectively. The 5′-monophosphorylated GGG fragment was added at a concentration of 250 nM or 2.5 μM. Lanes 1–6 correspond to time points of 0, 2, 5, 15, 60 and 120 min, respectively. The labelling is as in Fig. 1.

RNase G can moderate the maximal rate of cleavage. A Michaelis–Menten analysis of cleavage of precursor M1 RNA, the catalytic subunit of RNase P, suggests the same for RNase E [9]. Given members of the RNase E/G family form multimers, these results add weight to the suggestion that a single-stranded region could cooperate in binding enzyme that cleaves at another position. As mentioned above, the need for cooperative binding may increase the selectivity of members of the RNase E/G family. Moreover, should cleavages that initiate mRNA degradation require recognition of a single-stranded segment(s) within a protein-coding region this would allow translation to directly influence these enzymes by making them compete with ribosomes for binding to mRNA. This would minimise the cleavage of individual transcripts that are being highly translated, while ensuring that translationally defective or blocked mRNAs are removed rapidly [28].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.156.

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