Both N-terminal catalytic and C-terminal RNA binding domain contribute to substrate specificity and cleavage site selection of RNase III

Christian Conrad, Elena Evguenieva-Hackenberg, Gabriele Klug*

Institut für Mikro- und Molekularbiologie der Justus-Liebig-Universität Giessen, Heinrich-Buff-Ring 26–32, 35392 Giessen, Germany

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Abstract The double-stranded RNA-specific endoribonuclease III (RNase III) of bacteria consists of an N-terminal nuclease domain and a double-stranded RNA binding domain (dsRBD) at the C-terminus. Analysis of two hybrid proteins consisting of the N-terminal half of *Escherichia coli* RNase III fused to the dsRBD of the *Rhodobacter capsulatus* enzyme and vice versa reveals that both domains in combination with the particular substrate determine substrate specificity and cleavage site selection. Extension of the spacer between the two domains of the *E. coli* enzyme from nine to 20 amino acids did not affect cleavage site selection. © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: RNase III; Double-stranded RNA binding domain; Hybrid protein; RNA processing; *Rhodobacter*

1. Introduction

RNase III (EC 3.1.26.3) is a double-strand-specific endoribonuclease that cleaves rRNA precursors in bacteria, yeast and humans during maturation of rRNA [1–4]. It is also involved in mRNA and small nuclear or small nucleolar RNA processing, RNA interference [1,5–8] or rRNA fragmentation occurring in many bacterial species [9,10]. The enzyme is active as a homodimer and each subunit contains two functional domains [11,12]: a C-terminal double-stranded RNA binding domain (dsRBD) is the mediator of the observed dsRNA specificity, whereas the N-terminal catalytic part harbors a stretch of nine highly conserved amino acids, known as the RNase III signature box. The amino acids involved in formation of the active site have not been characterized in detail. Important residues include G48 and E117 of *Escherichia coli* RNase III, as well as two alanines (A216 and 217) within the dsRBD [13–16]. The structures of dsRBDs of different proteins have been solved [17,18], including a complex with dsRNA [19]. The dsRBD specifically recognizes and binds dsRNA and discriminates against single-stranded (ss) RNA, ss and dsDNA or DNA/RNA hybrids.

The structure of the *E. coli* RNase III dsRBD has been determined by nuclear magnetic resonance [11] and follows the general α_1-β_1-β_2-β_3-α_2 secondary structure of the dsRNA binding motif. Binding of proteins to RNA via the dsRBD seems to be sequence-unspecific [19]. Genetic and biochemical studies indicate that RNase III of *E. coli* can be considered a dsRNA binding enzyme carrying an endonuclease domain [12,20]. To investigate the modular organization of bacterial RNase III and to determine the role of the individual domains of the enzyme we exchanged the dsRBDs between RNases III of *Rhodobacter capsulatus* and *E. coli*. The endoribonucleases III of these organisms have been shown to possess different cleavage specificities on the one hand [9,21] and to have a similar size and sequence (overall amino acid identity of 34%) on the other hand [3,22], which makes them ideal candidates for the construction of chimeric enzymes. Furthermore we have constructed a variant of the *E. coli* enzyme with additional amino acids inserted in the linker region between the N-terminus and the dsRBD which has been tested for functionality and cleavage site selection.

2. Materials and methods

2.1. Construction and overexpression of hybrid RNases III

To generate fragments corresponding to the N-terminal and the C-terminal (dsRBD) part of the RNases III we PCR-amplified the regions encoding the RNase III N-terminus extending from position 1 to 438 of the *E. coli rnc* gene (strain JM109, Stratagene), and from position 1 to 456 of *R. capsulatus rnc* (strain 37b4, DSM 938). The dsRBD regions are located within bases 439–681 of *E. coli rnc* and at position 457–687 of the *R. capsulatus rnc* gene. For construction of the *E*/*R*-hybrid protein the primers No. I *Sac* and No. I *Sal* were used for the N-terminal part (*E. coli*) and the two primers No. IV *Sal* and No. IV *Pst* for the C-terminal dsRBD-region (*R. capsulatus*). For construction of the *R*/*E*-hybrid the primers were No. III *Sac* and No. III *Sal* for the N-terminus (*R. capsulatus*), as well as No. II *Sal* in combination with No. II *Pst* (C-terminus, dsRBD of *E. coli*). The sequences of the PCR primers were as follows (restriction sites underlined):

- No. I *Sac*: 5′-CCGCC<u>GAGCTC</u>ATGAACCCCATCGTA-ATT-3′; 29 nt; *Sac*I site;
- No. I *Sal*: 5′-GGGGG<u>GTCGAC</u>TTCGTCCAAACGAG-TTTG-3′; 29 nt; *Sal*I site;
- No. II *Sal*: 5′-GGGGG<u>GTCGAC</u>ATTAGCCCAGGCG-ATAAA-3′; 29 nt; *Sal*I site;
- No. II *Pst*: 5′-GGGGG<u>CTGCAG</u>TCATTCCAGCTCCA-GTTT-3′; 29 nt; *Pst*I site;

*Corresponding author. Fax: (49)-641-99 35549.
E-mail address: gabriele.klug@mikro.bio.uni-giessen.de (G. Klug).

Abbreviations: dsRNA, double-stranded RNA; dsRBD, double-stranded RNA binding domain

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- No. III *Sac*: 5′-GGGGGGAGCTCATGAAAGTTGCGGCAGAC-3′: 29 nt; *Sac*I site;
- No. III *Sal*: 5′-GTCGTTGTCGACGGACTGGATCCG-3′; 24 nt; *Sal*I site;
- No. IV *Sal*: 5′-CAGTCCGTCGACAACGACGCC-3′; 21 nt; *Sal*I site;
- No. IV *Pst*: 5′-GGGGGCTGCAGTCATGCGCCTCTCTCCAA-3′; 29 nt; *Pst*I site.

The resulting PCR products were purified, cut with *Sal*I and ligated, thereby swapping the particular domains. The ligated and newly combined fragments were cut with *Sac*I and *Pst*I and cloned into the corresponding sites of the polylinker of the pQE-30 hexahistidine tag vector (Qiagen). For protein expression the vectors were propagated in *E. coli* M15[pREP4] cells (Qiagen) at 37°C using standard I medium containing ampicillin (200 μg/ml) and kanamycin (25 μg/ml). Overexpression of the hybrid RNases III was induced by adding IPTG at a final concentration of 1.5 mM when cells reached an OD_{600} of 0.8. After continued incubation at 37°C for 2 h, the cells were harvested by centrifugation at 5000×*g* and stored at −70°C.

2.2. Cloning and overexpression of the N-terminal domains and dsRBDs of E. coli and R. capsulatus RNases III

The primers No. I *Sac* and No. I *Sal* (see above) were used for amplification of the *E. coli* N-terminus. The coding region for the *R. capsulatus* N-terminus was amplified using the primers No. III *Sac* and No. III *Sal* (see above). The *Rhodobacter* dsRBD was generated using the primers *Rc*dsRBD*Bam*HI (5′-CAGTCCGGATCCAACGACGCCCGC-3′; 24 nt; *Bam*HI site underlined) and No. IV *Pst* (see above), for amplification of the *E. coli* dsRBD the primers *Ec*dsRBD*Bam*HI (5′-GCGCGGATCCATTAGCCCAGGCGATAAA-3′; 28 nt; *Bam*HI site underlined) and No. II *Pst* (see above) were used. The resulting PCR products were ligated into pQE-30 as described before. Induction of protein expression by IPTG was carried out as described above.

2.3. Cloning and overexpression of the E. coli RNase III variant containing an insertion

We followed the approach used for the construction of the hybrid RNases III, this time fusing the N-terminal part of the *E. coli* enzyme to its own dsRBD after the linker region between the N-terminus and the dsRBD (encoding nine amino acids) had been doubled using appropriate PCR primers. The resulting protein (*Ec**) thus contained an extended region of 18 amino acids (plus two additional amino acids encoded by the six bases of the *Hin*dIII restriction site) separating the dsRBD and the N-terminal part of the enzyme. The following primers were used for PCR:

Amplification of the region encoding the N-terminal part of *E. coli* RNase III: No. I *Sac* (see above) and No. I link *Hin*d (5′-CCCCAAGCTTATCTTTTTGTTTATCGCC-3′; 28 nt, *Hin*dIII site underlined).

Amplification of the region encoding the dsRBD of *E. coli* RNase III: No. II Pst (see above) and No. II link *Hin*d (5′-CCCCAAGCTTATTAGCCCAGGCGATAAA-3′; 28 nt, *Hin*dIII site underlined). The resulting PCR products were purified, cut with the appropriate enzymes and ligated into pQE-30 as described before.

2.4. Purification of RNases III and isolated RNase III domains

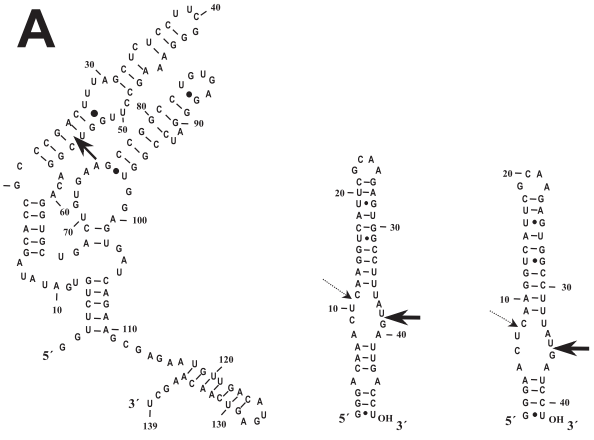
All recombinant proteins (possessing a hexahistidine tag) were purified as previously described [21]. *E. coli* RNase III was purified from the overexpressing *E. coli* strain HMS174(DE3)/pET-11a(*mc*) as previously described [23].

2.5. In vitro transcription of RNAs

The DNA template for the synthesis of the *Rc* mini RNA has already been described previously [21], and so have the N26 and N44 (R1.1[LSΔ6]) RNAs [24,25].

PCR amplification of the helix 9 regions between positions 109 and 205 of 23S rDNA (*E. coli* numbering) was performed as previously described [9].

The pUC18 plasmid carrying the DNA sequence of the *Rc* mini substrate located behind a T7 promoter was linearized with *Hin*dIII to enable run-off transcription [21]. As templates for N26 and N44 we used oligonucleotides with an annealed 18-mer promoter oligonucleotide [24,25]. In vitro transcription of the 23S rRNA helix 9 amplificates was performed as previously described [9,21]. The secondary structures of the *Rc* mini, N26 and N44 RNAs used as substrates for the different RNase III enzymes are shown in Fig. 1. RNA structures were predicted by computer analysis using the *mfold* program [26]. In addition, the structures of the N26 and N44 RNAs were analyzed experimentally [25]. The helix 9 substrates derived from 23S rRNA have been shown before [9].



substrate	*Rc* mini RNA	N26 RNA	N44 RNA
length [nt]	139	47	41
fragments generated: primary site	54 [5′] + 85 [3′]	38 [5′] + 9 [3′]	35 [5′] + 6 [3′]
fragments generated: secondary site	(not determined)	38 [5′] ⩾ 28 + 10	35 [5′] ⩾ 28 + 7

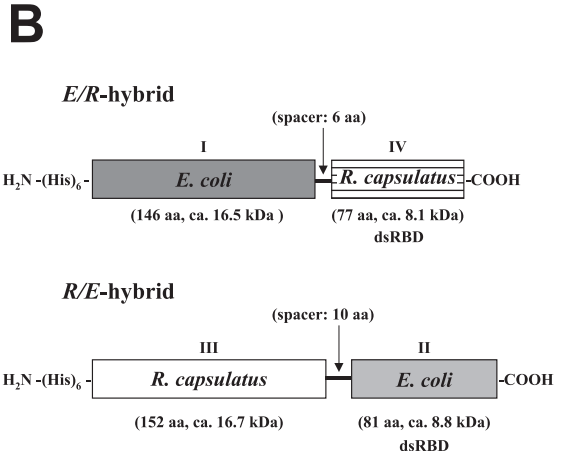


Fig. 1. A: Sequences and proposed secondary structures of three RNA substrates used in this study. The processing sites for RNase III are indicated by arrows: bold arrows: primary (3′) site; smaller arrows (dotted line): secondary (5′) site. The table summarizes the fragment sizes generated by wild-type RNase III cleavage of the particular RNAs. Under standard conditions the *Rc* mini RNA is only cleaved by *R. capsulatus* RNase III and the N44 RNA is only cleaved by *E. coli* RNase III. B: Schematic representation of the two hybrid RNases III. The sizes and the molecular weights (kDa) of the different domains are indicated (aa = amino acids).

2.6. Enzymatic assays

Cleavage assays were performed using in vitro transcribed, internally labelled RNA substrates, as described above. Standard cleavage buffer was 30 mM Tris–HCl, pH 7.5; 10 mM $MgCl_2$ or MgOAc, 130 mM KCl, 5% glycerol. Incubation was carried out at 37 or 32°C. Changes of the standard conditions are indicated. For each assay 5000 cpm of RNA substrate was used, the reactions were stopped by the addition of formamide containing dye and placed on ice. Reaction products were incubated at 65°C for 3 min and analyzed on a 10% PAA/7 M urea gel. Bands were detected by autoradiography.

2.7. Mapping of RNA 5′-ends by primer extension

To determine the exact RNase III cleavage position at the 3′-processing site in helix 9 of 23S rRNA we used primer extension analysis as described in [9]. After ethanol precipitation, the processed RNA substrate was treated as previously described [27]. Radioactively labelled sequencing reactions of the cloned DNA template were loaded on the same gel to map the position of the cleavage site for RNase III.

3. Results

3.1. Substrate specificity of RNase III hybrid enzymes

The different RNase III variants as well as the individual domains (N-termini and dsRBDs from *E. coli* and *R. capsulatus*) were purified to apparent homogeneity (not shown) and used for the enzymatic assays. The two hybrid RNases III, *E*/*R* and *R*/*E*, are represented schematically in Fig. 1B.

To analyze the cleavage behavior of the hybrid RNases III we used three different in vitro RNA substrates (Fig. 1A). N26 RNA (47 nt) derived from the phage T7 R1.1 RNase III processing signal is cleaved by both wild-type enzymes in a similar manner under standard conditions (130 mM KCl, not shown) as well as at lower (25 mM) and higher (180 mM) salt concentrations (Fig. 2). The same specificity is observed for the two hybrid proteins. The resulting fragments after cleavage of the N26 RNA correspond to the products that have been described previously [21,23–25]. This substrate can therefore be used as a standard substrate to test the enzymatic activity of both hybrids.

The *Rc* mini RNA (139 nt; Fig. 1A) is derived from the extended helix 46 processing site of RNase III in *R. capsulatus* rRNA and has previously been described [21]. RNase III of *R. capsulatus* specifically cleaves the *Rc* mini RNA while purified *E. coli* RNase III shows no detectable enzymatic activity with the same substrate under standard conditions (not shown) [21]. Under low salt conditions, even the *E. coli* enzyme cleaves the *Rc* mini RNA, a behavior that has been noted before [21]. This RNA is cleaved by the *E. coli* and *Rhodobacter* enzymes at additional positions when the salt concentration is decreased, resulting in fragments of approximately 60 nt and smaller fragments (Fig. 2, lanes 20 and 22).

The two hybrid RNases III do not significantly cleave the *Rc* mini RNA under standard conditions (not shown). Lowering the salt concentration enables processing of the *Rc* mini substrate by the *E*/*R* hybrid, and, to a lesser extent, by the *R*/*E* hybrid (compare Fig. 2, lanes 24 and 26). The *E*/*R* hybrid seems to exhibit a higher activity which might be due to enhanced substrate binding mediated by the C-terminal dsRBD of *R. capsulatus* RNase III.

The N44 (R1.1[LSΔ6]) RNA (41 nt, Fig. 1A) is cleaved by the *E. coli* RNase III but not by *Rhodobacter* RNase III under standard conditions [21]. The *E*/*R* hybrid is able to process this RNA while under the same conditions the *R*/*E* hybrid does not cleave the N44 RNA (Fig. 2). According to this observation the particular N-terminus of RNase III determines the enzyme's ability to process the N44 RNA. At low salt concentration, the *Rhodobacter* enzyme cleaves this RNA predominantly producing fragments of about 28 and 33 nt (Fig. 2, lane 13).

It has been noted before that otherwise unreactive RNAs could serve as RNase III substrates at low salt concentrations [23,28,29]. The enzymes exhibit a higher preference for secondary site cleavage at low salt concentration, especially the *R. capsulatus* RNase III (Fig. 2, lanes 2 and 4), a behavior which has been observed before [21,23]. Processing activity of hybrid and wild-type RNase III significantly decreases as salt concentration increases (Fig. 2).

For the various experiments represented in Fig. 2 different concentrations of salt for the different types of enzymes (wild-type or hybrids) have been used. Because of the increased salt sensitivity of the hybrids, the highest salt concentration used in the cleavage assays with these enzymes was not raised above 180 mM KCl for the N26 RNA and not above 150 mM KCl for N44 and *Rc* mini RNAs. All of the employed substrate RNAs can be cleaved by the different RNases III used in this study at salt concentrations lower than about 50 mM KCl (Fig. 2). One exception is the N44 RNA, the shortest molecule in this set of substrates: while RNases III of *E. coli*, of *R. capsulatus* and the *E*/*R* hybrid cleave the N44 RNA under low salt conditions, the *R*/*E* hybrid remains inactive towards this substrate, even at a low KCl concentration of 25 mM (Fig. 2, lane 17).

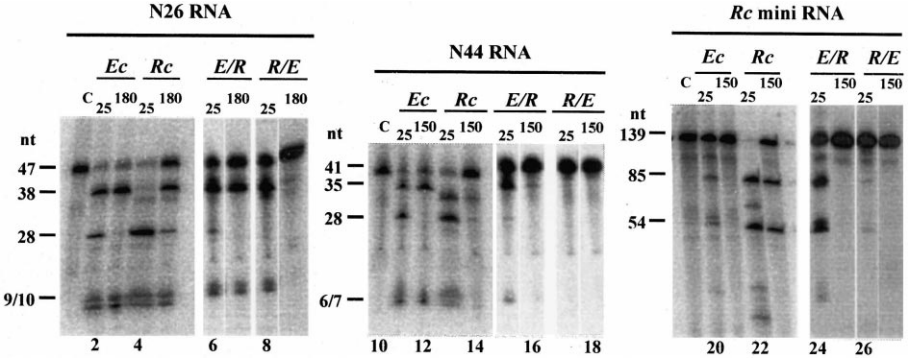


Fig. 2. Incubation of different RNAs (see Fig. 1, approx. 5000 cpm per lane) with *E. coli* (*Ec*), *R. capsulatus* (*Rc*), and hybrid (*E*/*R*, *R*/*E*) RNases III (30 nM dimer each; 3 min incubation time at 37°C) at different salt (KCl) concentrations. The KCl concentration (mM) used is indicated for each lane. Fragment sizes (nt) are indicated on the side of each panel. C: control (uncleaved RNA).

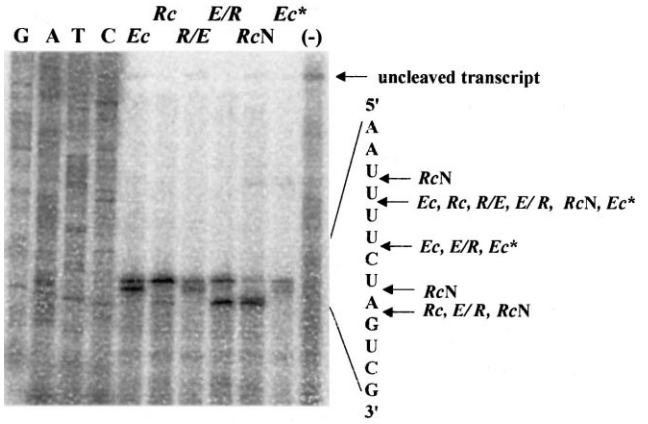


Fig. 3. Primer extension analysis of cleaved *B. henselae* transcript. RNA was incubated for 15 min at 130 mM KCl with *E. coli* (*Ec*) and *R. capsulatus* (*Rc*) RNases III, and for 50 min at 50 mM KCl with hybrid (*E/R, R/E*) RNases III and with the *Ec** RNase III variant. These assays were performed in the presence of 10 mM $MgCl_2$ at 37°C. The cleavage with the *Rc* N-terminus (*Rc*N) was performed for 15 min at 37°C in buffer without KCl containing 1 mM $MnCl_2$. (−): control primer extension reaction using uncleaved transcript. G, A, T, C each refer to the corresponding nucleotide of the DNA template. A part of the RNA sequence, complementary to the determined DNA sequence, is shown. The detected 5′-ends are marked by arrows.

3.2. Cleavage site selection by RNase III variants

It was previously shown that *Rc* RNase III and *Ec* RNase III often cleave different scissile bonds when they process certain substrates derived from precursor 23S rRNA of some α-proteobacterial strains [9]. These substrates include helix 9 of 23S rRNA, in which intervening sequences (IVSs) are localized. The IVSs are processed by RNase III leading to fragmentation of the 23S rRNA molecule in the 5′-region.

In order to compare the exact cleavage sites used by the hybrid RNases III with those used by the wild-type enzymes we performed primer extension experiments. Four different substrates derived from pre-23S rRNA of the bacterial strains *R. capsulatus* 37b4, *Rhodopseudomonas palustris* 5D, *Bartonella henselae* ATCC 49882, and *Rhizobium etli* Viking I were used [9]. All substrates with the exception of the one derived from *B. henselae* ATCC 49882 were cleaved by the *E/R* hybrid mostly in the same way as observed for *E. coli* RNase III. Cleavage by the *R/E* hybrid was mostly the same as for *Rhodobacter* RNase III. In some cases the hybrid RNases III cleaved additional scissile bonds which are not cleaved by the wild-type enzymes (not shown). Interestingly, the transcript derived from *B. henselae* ATCC 49882 was cleaved by the hybrid RNases in an opposite manner (see Section 4 for further comments). In this case, the C-terminal part of the RNase III determined cleavage site selection (Fig. 3).

3.3. Incubation of RNAs with individual domains of RNases III

It has been noted before that the isolated N-terminal domain of *E. coli* RNase III was able to cleave RNA under special (low salt, Mn^{2+}) conditions [1,20]. With the *Rhodobacter* RNase III N-terminus we were able to show this cleavage activity, using N26 RNA and 1 mM $MnCl_2$ instead of $MgCl_2$ at low salt concentration. This substrate was processed by the *Rc* N-terminus producing a different cleavage pattern than the wild-type enzymes (Fig. 4A). Using the *Bartonella* RNA as a substrate the specificity of the *Rc* N-terminus closely resembled that of the wild-type RNase III from *Rhodobacter* (Fig. 3). The same is true for a transcript derived from 23S rRNA of *Rhizobium leguminosarum* ATCC 10004 (described in [9], data not shown).

The isolated dsRBDs of the RNases III from *R. capsulatus* and *E. coli* did not exhibit cleavage activity. As has been

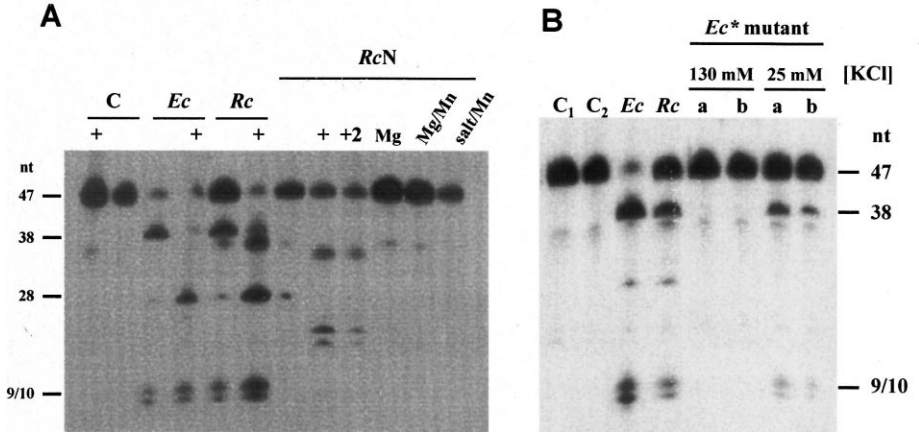


Fig. 4. A: Incubation of N26 RNA (approx. 5000 cpm/lane) by wild-type RNases III (*Ec*, *Rc*; 30 nM dimer each) and the *Rhodobacter* RNase III N-terminus (*Rc*N; 60 nM dimer) under different conditions. For the wild-type enzymes, the incubation time was 5 min, for the *Rc*N 15 min at 37°C. The RNAs were incubated in standard assay buffer (10 mM $MgCl_2$; 130 mM KCl), the particular changes in buffer composition are indicated according to the following abbreviations: a '+' above any lane indicates that incubation of the particular RNA was carried out in buffer lacking KCl, containing 1 mM $MnCl_2$ instead of $MgCl_2$. '+2' indicates that 2 mM $MnCl_2$ was added. C: control (no enzyme added). *Ec, Rc*: incubation with *E. coli* and *R. capsulatus* RNase III, respectively. *Rc*N: incubation with the *Rc* N-terminus. Mg: incubation with *Rc*N in buffer without KCl, this time containing 10 mM $MgCl_2$. Mg/Mn: incubation with *Rc*N in buffer lacking KCl and containing both 10 mM $MgCl_2$ and 1 mM $MnCl_2$. salt/Mn: incubation with *Rc*N in buffer containing 130 mM KCl and 1 mM $MnCl_2$. Fragment sizes (nt) are indicated. B: N26 RNA (approx. 5000 cpm/lane) incubated with wild-type RNases III (*Ec, Rc*) and with the *E. coli* RNase III mutant (*Ec**) containing additional amino acids between the N-terminus and the dsRBD. Enzyme concentrations were 30 nM dimer. C_1: control, incubated in standard assay buffer C_2: control, incubated in standard buffer lacking KCl. *Ec, Rc*: RNA incubated with *E. coli* RNase III and *Rhodobacter* RNase III, respectively. *Ec** mutant: incubated with the *Ec** RNase III variant in buffer containing 10 mM $MgCl_2$, KCl concentrations [mM] are indicated. a: incubation time 5 min, b: incubation time 3 min (37°C). Fragment sizes (nt) are indicated.

reported for the yeast enzyme [30], mixing the isolated N-termini and the dsRBDs did not reconstitute cleavage activity of the bacterial enzymes under standard conditions.

3.4. Extending the spacer region between the N-terminal and C-terminal domains of E. coli RNase III does not alter cleavage specificity

Recently it has been reported that exchanging the dsRBDs between the RNA-specific adenosine deaminase ADAR1 and the interferon-inducible RNA-dependent protein kinase PKR influences the activity of the proteins, especially if the spacing between the RNA binding region and the catalytic domain had been changed [31]. *E. coli* RNase III contains a spacer region of nine amino acids, the *Rhodobacter* enzyme a spacer of six amino acids, the *E*/*R* hybrid has a spacer of six and the *R*/*E* hybrid one of 10 amino acids located between the N-terminus and the dsRBD of the enzyme. From our experiments we could not observe any influence of the particular spacer length on cleavage site selection (i.e. the hybrids did not show a substrate specificity corresponding to the spacer length of one of the wild-type proteins). However, to further test whether cleavage site selection by bacterial RNase III also depends on the distance between the dsRBD and the N-terminal catalytic part of the enzyme, we inserted additional amino acids within the linker region. Using the *E. coli* enzyme, the spacing between the dsRBD and the N-terminal region was increased from nine amino acids to 20 amino acids by duplication of the corresponding linker region (plus two amino acids encoded by the *Hin*dIII site, which was introduced by PCR). The insertion of the additional amino acids between the N-terminus and the dsRBD of RNase III decreases the enzymatic activity by increasing its salt sensitivity (as has been observed for the hybrid proteins). The N26 RNA used for these experiments is only cleaved at low salt concentrations. The selection of the cleavage site per se, however, is not influenced by the additional spacer amino acids (Fig. 4B). This was also investigated by primer extension analysis with the *Bartonella* substrate mentioned above. In vitro cleavage of this RNA did not exhibit differences between the *E. coli* wild-type enzyme and the *Ec** mutant containing the additional amino acids (Fig. 3).

4. Discussion

Our results clearly show that RNase III is indeed built of two separable and interchangeable domains. The modular structure of RNase III is emphasized by the following results: a standard substrate for RNase III (N26 RNA, see Fig. 1) can be cleaved by both hybrid RNases III at the same specific positions within this RNA (Fig. 2). This indicates the functionality of the hybrid enzymes, and shows that they do not need to be composed of their original two domains. Instead, the N-terminal part of *E. coli* RNase III is catalytically active carrying the dsRBD derived from *R. capsulatus* RNase III (*E*/*R* hybrid), and vice versa (*R*/*E* hybrid). Cleavage at the particular secondary site of the RNAs (indicated in Fig. 1A) seems to be slightly less with the two hybrids. This is perhaps due to a generally increased salt sensitivity of the chimeric proteins (Fig. 2).

The *Rc* mini RNA and the N44 RNA have already been shown to be cleaved with different specificities by the wild-type RNase III of *E. coli* and *Rhodobacter* [21].

The *E*/*R* hybrid and the *R*/*E* hybrid are only able to process the *Rc* mini substrate at low salt concentrations (compare Fig. 2, lanes 24 and 26). The higher activity of the *E*/*R* hybrid might be due to enhanced substrate binding mediated by dsRBD of *R. capsulatus* RNase III, whereas the observed fragment patterns resemble the patterns produced by the wild-type enzymes carrying the particular N-terminus. These results point in the direction of an interdependence of dsRBD and N-terminus of RNase III.

The inability of the *R*/*E* hybrid, carrying the N-terminal catalytic part of *Rhodobacter* RNase III, to cleave the N44 RNA, even at low salt conditions (Fig. 2), corresponds to the inability of the wild-type RNase III of *R. capsulatus* to cleave this substrate under standard conditions. Only addition of manganese allows the N44 RNA to be cleaved by the *R*/*E* hybrid (not shown). *E. coli* RNase III and the *E*/*R* hybrid on the other hand are both able to cleave the N44 RNA (Fig. 2). This indicates that both the *E. coli* and the *Rhodobacter* RNase III dsRBDs must be able to mediate binding to N44 under standard conditions. The N-terminus of *Rhodobacter* RNase III in the *R*/*E* hybrid (and in the *Rhodobacter* wild-type enzyme), however, fails to catalyze cleavage of the N44 substrate. The N-terminal part of *E. coli* RNase III contained within the *E*/*R* hybrid does cleave the N44 RNA (Fig. 2). In this case, the N-terminus determines the substrate specificity.

Our results regarding the in vitro cleavage of RNase III substrates derived from helix 9 of 23S ribosomal RNAs of various bacteria (not shown) provide further evidence for the interdependence of the RNase III N-terminus and the dsRBD in determining the position of the cleavage site. The exceptional behavior of the transcript derived from *Bartonella*, i.e. the dsRBD determining cleavage site selection, may be in part due to the different base composition and structure of this substrate. The helix 9 of 23S rRNA of *B. henselae* ATCC 49882 which contains the RNase III processing site possesses an AT content of 73% in contrast to the AT content of 50–55% found in the first 30 bp of helices 9 in *R. etli* Viking I, *R. palustris* 5D and *R. capsulatus* 37b4 [9]. In addition the *B. henselae* RNA possesses an extraordinarily long dsRNA region which might also contribute to the enhanced influence of the dsRBD.

Enzymatic activity of an isolated N-terminus of RNase III in vitro has until now only been mentioned for the *E. coli* enzyme [1]. According to the author, the dsRBD of *E. coli* RNase III is necessary for RNase III activity in vivo, but under certain unphysiological conditions in vitro the dsRBD is not needed for enzymatic activity. We could show cleavage activity for the isolated *Rhodobacter* RNase III N-terminus with N26 RNA under manganese conditions at low concentrations of KCl. The observed cleavage pattern, however, was different from the one produced by the full-length enzyme. Addition of magnesium ions (alone or in addition to $MnCl_2$) inhibited the cleavage that occurred in the presence of Mn^{2+} alone (Fig. 4A). In this case an excess of Mg^{2+} might be able to compete with Mn^{2+} for the binding site. Magnesium ions are obviously not able to promote RNA cleavage by the isolated N-terminus of *Rhodobacter* RNase III. The influence of magnesium and manganese ions on the catalytic mechanism of RNase III has recently been discussed in detail [13].

The transcripts derived from *Bartonella* (Fig. 3) and *Rhizobium* (not shown) are cleaved by the *Rc* N-terminus with

apparently the same specificity as by the *Rhodobacter* RNase III (Fig. 3), an observation that strongly argues against a mere enhancement of cleavage at particular hot spots. We conclude that the isolated N-terminus of RNase III is sufficient for specific cleavage of certain substrates in the presence of manganese and at low salt concentrations. Most interestingly, fusing a C-terminal dsRBD that originates from a RNase III of a different bacterium (like in the hybrid proteins), the cleavage site selection of the resulting chimeric enzyme is changed, at least with this particular substrate from *Bartonella*. Thus, a more complex interaction and interdependence between the N-terminus, the dsRBD and the particular RNA substrate determines the cleavage site.

For Rnt1, the RNase III homologue of *Saccharomyces cerevisiae*, the N-terminal part of the protein lacking the dsRBD does not seem to exhibit any enzymatic activity [30]. The same is true for the RNase III N-termini used in our study under standard conditions (i.e. with Mg^{2+}, lacking Mn^{2+}; 130 mM KCl), where they were catalytically inactive when expressed without their dsRBDs.

A variant of *E. coli* RNase III (*Ec**), containing additional amino acids inserted between the N-terminus and dsRBD of the enzyme, is able to cleave N26 RNA, although the efficiency is decreased. The specificity of this enzyme variant is not altered by the linker insertion (Fig. 4B). In addition, the substrate derived from *Bartonella* rRNA is also processed by the *Ec** mutant without notable changes in cleavage site selection compared to the *E. coli* wild-type enzyme (Fig. 3). Changing the distance between the N-terminal catalytic part and the dsRBD therefore influences the enzyme's activity rather than its specificity (in terms of cleavage site selection). Similar observations have been made for ADAR1 and PKR [31].

Recent data concerning the binding properties of the dsRBD [17–19] do not support the hypothesis that this module is directly responsible for any particular sequence specificity in addition to the selection of dsRNA. This strengthens the idea that the dsRBD alone does not directly determine the cleavage site, but facilitates binding to the dsRNA substrate in vivo and under physiological conditions in vitro, as has been discussed before. The catalytic N-terminal part of RNase III is probably brought into close proximity to the particular cleavage site by the enzyme's dsRBD. The selection of the exact scissile bond which will subsequently be cleaved is carried out by a more subtle interaction of the dsRBD, the particular RNA and the RNase III N-terminus, including the active site of the enzyme, which still remains to be further defined biochemically.

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