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RNase III: Genetics and Function; Structure and Mechanism

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Abstract

RNase III is a global regulator of gene expression in *Escherichia coli* that is instrumental in the maturation of ribosomal and other structural RNAs. We examine here how RNase III itself is regulated in response to growth and other environmental changes encountered by the cell and how, by binding or processing double-stranded RNA (dsRNA) intermediates, RNase III controls the expression of genes. Recent insight into the mechanism of dsRNA binding and processing, gained from structural studies of RNase III, is reviewed. Structural studies also reveal new cleavage sites in the enzyme that can generate longer 3' overhangs.

Keywords

global gene regulation; dsRNA binding; dsRNA processing; bacteriophage; CRISPR; dicer

INTRODUCTION

The RNase III proteins are Mg²⁺-dependent, double-stranded RNA (dsRNA)-specific endonucleases that are characterized by a nine-residue signature motif in their specialized endonuclease domain, the so-called RNase III domain (RIIID, see Figure 1a). The RIIID has also been abbreviated as endoND, NUCD, RNaseIII, RIII, III, etc.; however, RIIID is preferred, as it best describes its specificity (RIII for RNase III), completeness (D for domain), and clarity (versus the RNase III protein). The cleavages performed by RNase III enzymes generate 5' phosphoryl and 3' hydroxyl ends with a two-nucleotide (2-nt) 3' overhang in their dsRNA products (23, 61, 76, 91). They play roles in ribosomal RNA (rRNA) processing, posttranscriptional gene expression control (23, 61, 116), and defense against viral infection (27, 65, 91, 93, 106). The RNase III family includes bacterial RNase III and eukaryotic Rnt1p, Drosha, and Dicer (13, 36, 54), among which RNase III from

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Escherichia coli is the most comprehensively studied member (for other reviews, see 23, 30, 76, 77, 90). In this review, RNase III refers to *E. coli* RNase III unless otherwise specified.

THE GROWING RNase III FAMILY: DIVERSITY OF FORM AND FUNCTION

Since the initial discovery of *E. coli* RNase III some 45 years ago, RIIID domains have been found in a variety of proteins, ranging in length from ~140 (mini-III) to ~1,900 (Dicer) amino acid residues, that participate in a diverse array of functions. Proteins with this activity are universally conserved in all species of bacteria and eukaryotes. The simplest RNase III protein is the *Bacillus subtilis* Mini-III, which functions in the maturation of the 23S rRNA. The Mini-III protein consists of a single RIIID (Figure 1a) (86). *E. coli* and other bacterial RNase III proteins, in addition to the RIIID, also contain a dsRNA-binding domain (dsRBD) that allows RNase III to be multifunctional and act as either a processing endonuclease or a dsRNA-binding protein (18, 23, 26, 51, 77, 81).

RIIID-containing proteins found in lower eukaryotes tend to be more complex than their bacterial counterparts. Three distinct types of RNase III enzymes have been characterized in members of the budding yeast lineages, represented by Rnt1p from *Saccharomyces cerevisiae* (ScRnt1p), Dcr1 from *Kluyveromyces polysporus* (KpDcr1), and Dcr1 from *Candida albicans* (CaDcr1) (11). ScRnt1p is the only RNase III in *S. cerevisiae* (2), and it contains an uncharacterized N-terminal domain (NTD) that is followed by an RIIID and a dsRBD (Figure 1a); ScRnt1p is important in the processing and maturation of 35S precursor rRNA, small nucleolar RNAs (snoRNAs), and small nuclear RNAs (snRNAs) (1, 2, 20, 21). KpDcr1 is a noncanonical Dicer. It contains an NTD, a RIIID, and two dsRBDs and produces small interfering RNAs (siRNAs) that direct RNA interference (RNAi) (109). The domain structure of CaDcr1 is similar to that of KpDcr1; however, CaDcr1 is bifunctional. In addition to functioning as a noncanonical Dicer, it also catalyzes the processing and maturation of 35S precursor rRNA and snRNAs similar to ScRnt1p (11).

Even more complex RIIID-containing proteins are found in multicellular eukaryotes; these include the canonical Dicer and Drosha enzymes, which produce siRNAs and micro RNAs (miRNAs) that mediate RNAi, (12, 19, 66). Drosha catalyzes the initial processing of the stem loop (hairpins) from primary miRNAs (pri-miRNAs) into short, hairpin, precursor microRNAs (pre-miRNAs), and Dicer further processes the pre-miRNAs into miRNAs (67) that regulate more than half of all mammalian coding sequences by RNAi (40). Much of this RNAi-mediated regulation occurs as the miRNA binds to the 3' untranslated region (3' UTR) and regulates expression from those transcripts. Historically, RNase III was the first example of a regulator shown to bind at sites and control gene expression from beyond the coding sequence. This process, called retroregulation, is described in more detail below (48).

A typical canonical Dicer, such as the human enzyme (HsDicer) (Figure 1a), contains a DExD helicase domain, a DUF283 domain, a PAZ (piwi argonaute zwille) domain, two RIIIDs, and a dsRBD (12). Nonetheless, a fully functional Dicer from *Giardia intestinalis* (GiDicer) does not have the helicase domain or dsRBD, the exact significance for the presence or absence of these domains is not clear (69). Unlike Dicer, the Drosha N-terminal extension contains a P-rich (proline-rich) domain and an RS-rich (arginine+serine-rich)

domain, but similar to Dicer, it also contains two RHHs followed by a dsRBD. In vitro, both Dicer and bacterial RNase III can be used to produce siRNA cocktails, which are effective RNAi mediators (117, 119). Structurally, however, Dicer (~1,900 amino acid residues) is much more complicated than RNase III (226 residues) (Figure 1a). Therefore, RNase III has served as the principal model enzyme for evaluating the mechanistic details of basic RNase III activity.

THE RNase III GENE *rnc* AND THE *rnc* OPERON OF *Escherichia coli*

Hugh Robertson first detected RNase III by the presence of its double-strand specific endoribonuclease activity in *E. coli* extracts (91). Five years later, the first defective mutant in RNase III activity was isolated by a brute-force genetic approach using heavy mutagenesis and by screening many colonies for one that had lost this nuclease activity (59). A plasmid clone that complemented the mutant was isolated and sequenced, revealing the *rnc* gene that encodes the 25-kDa RNase III polypeptide (74, 108).

The *rnc* gene is the first gene in the *rnc* operon, which also contains the *era* and *recO* genes (3, 102). The *rnc* and *era* genes are not only expressed in the same operon, but their translation is coupled to ensure similar levels of expression. (9, 16, 102). The main substrates for RNase III in *E. coli* are the rRNA transcripts from the seven rRNA operons. The transcript from each of these operons contains the 16S rRNA, 23S rRNA, 5S rRNA, and tRNAs, which are transcribed as a unit and are processed by RNase III as transcription occurs (38, 120). The 16S and 23S rRNAs are each flanked by inverted repeat sequences that form two sets of stem structures with the 16S and 23S rRNAs looped out between the near perfectly paired stems of 36 and 28 base pairs (bp), respectively. RNase III cuts into the 36-bp and 28-bp stems to release precursors 16S and 23S, respectively. The timing of transcription and processing are closely coupled (38), and the coordination of transcription and RNase III processing are important for rapid folding of rRNAs and assembly of ribosome subunits (17). In view of the important role that RNase III plays in rRNA processing, one might expect that it would be essential for cell viability; however, it is not. In an *rnc* mutant defective for RNase III, the full-length ribosomal operon transcript is produced, which never occurs when RNase III is present (45). In the absence of RNase III, this full-length transcript is processed by other ribonucleases to generate 16S and 23S rRNAs, albeit more slowly. (28).

Bacterial operons often contain genes with related functions. This is true for Era and RNase III encoded by the *rnc* operon. Both are important for efficient ribosomal processing and maturation. Biochemical, genetic, and physical analyses have shown that there is a general requirement for Era in ribosome biogenesis and the coordination of cell growth and division (3, 16, 104). Crystal structure studies of *E. coli* Era reveal an N-terminal GTP-binding domain and a C-terminal KH RNA-binding domain (22). Era binds GTP, and the Era-GTP form of the protein binds in the 16S rRNA to the 10-nt residues GAUACCUCC containing the CCUCC complement to the Shine-Dalgarno sequence. Binding here ensures proper processing of the precursor 16S rRNA and the final maturation of the 30S ribosome subunit (104, 105). Processing by RNase III to release the precursor 16S rRNA occurs less than 40 nts beyond the anti-Shine-Dalgarno sequence and the Era binding site. Thus, the two

enzymes interact in close proximity on the rRNA and evidence suggests that RNase III cleaves and releases precursor 16S rRNA at about the same time Era binds (73, 104, 105); however, no protein-protein interaction has yet been observed. Unlike RNase III and Era, the RecO protein, encoded by the last gene in the *mnc* operon, has no known role in ribosome biogenesis but is instead involved in homologous recombination.

STRUCTURES THAT RNase III CLEAVES

Perfectly complementary dsRNA can be produced in vivo when the same segment of DNA is transcribed in both directions. Such dsRNA is efficiently cleaved by RNase III to yield the classical staggered cut with 2-nt 3' overhangs. Other targets of RNase III are intramolecular duplexes formed within the same RNA molecule, such as hairpin-loop (stem-loop) structures (23). Additionally, independent dsRNA hairpins can stack coaxially to generate a processed structure (37). In all cases, the helix must be of sufficient length, approximately 22 bp, to be bound and cleaved. The intramolecular stem-loop structures are usually not perfectly complementary and often have one or more mismatched base pairs or bulges in the stem structure. Depending on the location of these irregularities, they may cause RNase III to cleave only one or the other strand of the RNA stem, or prevent cleavage altogether, while still allowing RNase III to bind (18, 92). Thus, the ability to bind, to generate a single-strand nick, or cause a double-strand break by RNase III depends partly on base pairing of the stem-loop. The Nicholson laboratory has identified specific positions and types of mismatches within a well-characterized stem-loop structure that either inhibit binding of RNase III or allow binding but prevent cleavage. Systematic changes at each site along the duplex also revealed a hierarchy of sites with different degrees of sensitivity to cleavage by RNase III (85).

Cleavage conditions for these natural stem-loop structures are, for the most part, similar to cleavage conditions for long duplex RNA when compared in vitro (29). There is one example known in which a natural target site of RNase III in the 10Sa RNA (transfer-messenger RNA) in vivo was shown to require Mn^{2+} instead of Mg^{2+} for cleavage in vitro (98). In general, binding and processing can be stimulated or inhibited by changing ionic conditions and the choice of divalent cation.

THE WAYS IN WHICH RNase III IS REGULATED

Although the promoter activity of the *mnc* operon appears to be unregulated, there is a wealth of posttranscriptional regulation (102). Most directly, RNase III cleaves a stem-loop in its own 5' UTR, causing the mRNA to become vulnerable to degradation by other ribonucleases. This autoregulation reduces the mRNA level approximately fivefold and thereby influences the expression of both RNase III and Era (9, 71). Autoregulation is possible because the amount of RNase III protein in the cell is limited, representing less than 0.01% of total protein (9). Thus, RNase III protein can be competitively titrated away from its own transcript by its major substrate, rRNA (31, 78). Titration occurs as the transcription rate of rRNA reaches more than 80% of total transcription in the cell under rich media conditions (34). When this happens, the autoregulatory effect helps RNase III levels rise to meet the need of processing this rRNA.

In addition to RNase III autoregulation, *mnc* and *era* expression is also regulated by cellular growth rate. Growth-rate regulation affects ribosomal protein genes, the rRNA operons, and other functions involved in the biosynthesis of ribosomes (see 55 and references therein). Cells grown in a minimal medium produce 5- to 10-fold less RNase III and Era than cells grown in a rich broth medium (16, 114). Unlike the rRNA operons and most other genes, in which growth control is regulated at the level of transcription (55), here the regulation is at the level of translation (16). This growth control regulation occurs in the presence or absence of RNase III function, and thus is clearly distinct from RNase III processing and autoregulation. The mechanisms and functions responsible for this growth rate translation control are not known. The level of RNase III not only drops in cells undergoing a switch to poorer growth media, but also occurs when cells in a rich medium enter stationary phase; it is not yet known if this stationary phase reduction is related to growth-rate control or whether it occurs at the level of translation (58).

Different forms of posttranslational regulation modulate RNase III activity. The first type described was a phosphorylation of RNase III within a few minutes after infection by bacteriophage T7. The phage produces a serine/threonine-specific protein kinase (the product of the 0.7 gene of T7) that modifies serine residues of RNase III, resulting in approximately a fourfold stimulation of RNase III activity (72, 89). Increased RNase III activity is advantageous for T7 lytic development and stimulates processing and maturation of several early and late T7 transcripts (33).

E. coli crude extracts contain proteins that inhibit RNase III processing activity (70); one of these proteins, YmdB, is increased by the stress of cold shock. YmdB acts by binding to RNase III monomers precluding the formation of catalytically active RNase III homodimers (58). Yet, while catalytically inactive, the heterodimer retains the ability to bind dsRNA substrates. This residual binding ability is reminiscent of certain catalytic mutants of RNase III that bind dsRNA but do not cleave (see below) (13, 26, 100, 101).

Another environmental inhibitor of RNase III is osmotic stress, which also downregulates the activity of RNase III, independent of YmdB (97). In this case, the mechanism and inhibitory factors remain unknown. It may be among the other as-yet-uncharacterized multicopy plasmid clones identified as reducing RNase III activity in vivo (58).

Far less is known about the regulation of RNase III family members in organisms other than *E. coli*. In human cells, the CLIMP-63 membrane protein of the endoplasmic reticulum binds Dicer and stabilizes it to enhance its catalytic activity (84). A global regulatory protein like Dicer is almost certain to have more posttranslational interactions in response to different cellular environments.

THE WAYS IN WHICH RNase III REGULATES GENE EXPRESSION

Two decades ago we speculated that RNase III could be a global regulator with many cellular targets (23). This has now been confirmed by genome-wide analyses. As determined by polyacrylamide gel analysis, approximately 10% of all proteins in the cell are either over- or under-expressed in an *mnc* mutant (47). A more recent analysis using tiling microarrays

produced similar results, showing that approximately 12% of all mRNAs are affected by a mutant defective in RNase III, with 9% being increased and 3% decreased in abundance (99). These studies had an inherent bias because a mutant completely defective in RNase III activity retards growth relative to wild type, and poor growth itself causes changes in gene expression. A more refined approach utilized strains with altered levels of RNase III that still allowed normal growth of the bacteria (97). By reducing or increasing RNase III levels tenfold relative to wild type, 87 genes were shown to be upregulated and 100 genes were downregulated by RNase III. Hence, for the first time a quantified effect of different RNase III levels on global gene expression was tested. We have described in the previous section several ways in which RNase III levels change in a dynamic bacterial environment; such changes affect the expression of many genes.

RNase III can affect gene expression in a variety of different ways. For instance, processing by RNase III can change the structure of an mRNA so as to promote its degradation and thereby reduce gene expression. Alternatively, processing can alter the structure of an mRNA such that it is translated more efficiently by ribosomes. Cleavage of an mRNA by RNase III can be 5' to the coding sequence, 3' to the coding sequence, or within the coding sequence. The structures cleaved can be simple dsRNA stem-loops within the message, or they can be dsRNA structures created between the mRNA and small regulatory RNAs (sRNAs) (23, 49, 103). Thus, there are several ways in which RNase III processing controls mRNA gene expression. Below we describe some specific examples of such events and later examine other, more complex control circuits involving RNase III.

RNase III Can Either Activate or Inhibit Gene Expression by Cleavage of the 5' Untranslated Region

We have already described how RNase III cleaves its own mRNA, and thereby inhibits its expression in an autoregulatory feedback loop (9). The cleavage of similar stem-loop structures near the 5' ends of the mRNAs also inhibits the expression of downstream genes in the *pnp* and *metY* operons. These base-paired structures protect their mRNAs from degradation by masking free 5' ends (35). Processing by RNase III removes this protection and renders them susceptible to nucleases such as the single-strand-specific endonuclease RNase E, thereby reducing the half-life of these mRNAs (9, 87, 88).

RNase III can also activate gene expression by cleavage of stem-loop structures. In this case, the dsRNA structure occludes the ribosome-binding site and inhibits ribosome access for translation initiation; cleavage by RNase III opens the structure to allow efficient ribosome binding. The *N* and *0.3* genes expressed early in infection by phages λ and T7, respectively, are examples in which RNase III processing activates translation (23, 32, 57). Some bacterial genes can also be translationally upregulated by RNase III 5' processing. The *adhE* mRNA produces ethanol dehydrogenase, which is required during anaerobic growth on glucose. Translation of the *adhE* message requires processing by RNase III to remove a stem-loop structure that occludes its ribosome-binding site. This control on expression of the dehydrogenase is so tight that in the absence of RNase III, anaerobic growth is impossible when glucose is the sole carbon source (8).

Different classes and positions of structures within the 5' noncoding region of mRNAs determine whether RNase III processing causes a negative or positive effect on gene expression. For some transcripts, such as the *rnc* operon, removing 5' stem structures reduces mRNA stability; for other transcripts, such as the phage *N* and *O.3* genes, mRNA stability remains largely unchanged but ribosome binding is enhanced because steric overlap of the dsRNA stem on the ribosome-binding site is eliminated by stem cleavage. Steric effects of the stem on ribosome binding are not relevant for the *rnc*, *pnp*, or *metY* operon because the ribosome-binding site is located far downstream of the stem structures. Another major difference is that the strength of the ribosome-binding sites is much weaker for the *rnc* and *pnp* operons as compared with the phage genes. mRNAs containing weaker translation initiation sites are known to be more prone to mRNA degradation by the 5' processive endonuclease RNase E, but they can be protected by upstream base-paired structures that block RNase E. mRNAs with strong ribosome initiation sites are quite resistant to RNase E because ribosomes bind rapidly and protect them from degradation (68). Thus, RNase III cleavage only exposes the mRNA of weak translation initiation sites to RNase E decay.

Retroregulation: Control of Gene Expression by RNase III from the 3' Untranslated Region

Posttranscriptional regulation of gene expression from a 3' UTR is an important mechanism of gene regulation from bacteria to mammals that utilizes specific RNA-binding proteins and miRNAs to repress or activate expression (112). The first description of gene regulation from a 3' UTR was that of the *int* gene in bacteriophage λ by RNase III. At that time, the process was called retroregulation (48, 51, 52, 94, 95).

Two different promoters *pL* and *pI* transcribe the *int* gene at different stages of λ development, but only transcripts from *pI* are translated into the Int protein. RNA polymerase transcribing from the major *pL* promoter following λ lytic infection is modified by the λ N protein, which causes the polymerase to read through transcription terminators. This transcription from *pL* reaches the *int* gene and passes through *int* as well as the transcription terminator *tI* beyond *int*. This read-through of *tI* generates a 22-bp stem-loop structure that is cleaved by RNase III. The cleaved *int* mRNA is rapidly degraded by 3' exonucleases, preventing Int expression and integration of phage λ DNA into the bacterial genome during lytic development (52, 94).

Transcription of *int* from the second promoter, *pI*, just upstream of the *int* gene, is activated as part of a response to establish the λ phage-immune, lysogenic state. The N antiterminator protein does not modify this transcript, and polymerase terminates at *tI*. The terminated transcript does not form the full-length 22-bp stem-loop; therefore, it is not cleaved by RNase III, and it remains stable and generates high levels of Int protein for integration of phage DNA into the bacterial genome to establish the prophage state of the lysogen (95).

RNase III Activates Gene Expression by Processing the 3' Untranslated Region

Several early transcripts of bacteriophage T7 are unusually stable, with half-lives of ~20 min. The 3' ends of these transcripts are generated by RNase III cleavage, but these ends are special in that cleavage occurs only on the distal strand of the dsRNA stem. Thus, the cleavage product contains the T7 mRNA with a partial 3' stem-loop structure that

presumably protects the mRNA from processive exonucleases (33). If these particular RNase III sites are cloned into positions downstream of a foreign mRNA, they can stabilize the mRNA and increase gene expression (82). It is also possible that RNase III plays an active role in this stabilization by remaining bound after cleavage.

Binding by RNase III Can Exert Control on Gene Expression

Although RNase III typically cleaves one or both strands of an RNA duplex after binding, there are instances in which RNA binding occurs without cleavage. This is another manner in which RNase III may regulate gene expression. We have described the cellular protein YmdB, which by interacting with RNase III inhibits cleavage activity but not dsRNA-binding activity (58). As yet, no regulatory effect has been ascribed to the YmdB/RNase III heterodimer complex. However, there are examples in which wild-type RNase III protein appears to regulate gene expression in phage λ merely by binding dsRNA (51, 81).

The expression of the *cIII* gene of λ is stimulated by the presence of RNase III (7). Uniquely, two overlapping and mutually exclusive stem-loop structures form in the region of the *cIII* ribosome-binding site and extend 10 bases upstream of the AUG codon to 34 bases within the *cIII* coding sequence. Structural studies have shown the presence of both forms of the RNA in a state of equilibrium (6). RNase III does not cleave either of these RNA structures in vivo nor does it affect mRNA stability. One of the structures occludes the ribosome-binding site and blocks *cIII* translation; the other structure reveals the binding site and can activate *cIII* translation. Although there is no cleavage in vivo, RNase III is able to cleave the activating structure under special conditions in vitro, indicating the ability to bind that structure (5). Mutations that destroy only the inhibitory RNA structure become independent of RNase III for *cIII* expression, and conversely, mutations that destroy the activating structure are defective for *cIII* expression even in the presence of RNase III. This suggests that RNase III binds the activating structure, thereby favoring its formation and increasing *cIII* gene translation (5).

As we mentioned in the previous section, RNase III may remain bound after cleavage of terminal stem-loop structures of early T7 genes and stabilize their mRNA. The *int* gene transcript, terminated at *tI*, has a terminal stem-loop structure and is very stable, producing high levels of Int protein. In an *int* mutant, this terminated transcript becomes unstable, and Int protein expression is reduced. One explanation is that, although it cannot cleave the shortened terminator structure, RNase III still binds to it and protects it from 3' exonucleases (51, 95). Thus, λ CIII and λ Int protein expression appear to be stimulated by wild-type RNase III binding to structures near their coding regions. This possibility still needs to be tested by showing that an RNase III mutant protein with binding but not cleavage activity exerts the same effect as wild-type RNase III.

RNase III Can Cleave within Coding Sequences

Translation, which is closely coupled to transcription in bacteria, would be expected to preclude the formation of RNA structures in the coding regions of mRNAs and inhibit RNase III entry. Thus, it is not surprising that there are few examples of documented RNase III cleavage events within coding sequences. Those that exist may be special examples of

gene regulation. An example is found in *Streptomyces coelicolor*, where RNase III directly cleaves a stem-loop structure in its own coding sequence as a mechanism of autoregulation (118). Another example is found in *E. coli*, where the coding sequence of the *arfA* gene is cleaved by RNase III. It is this truncated transcript that generates the functional ArfA protein (44). ArfA functions to remove ribosomes stalled within a coding sequence, including those ribosomes at the ends of RNase III-truncated messages. RNase III cleavage within a coding sequence depends on levels of RNase III and ribosome translation rates across such coding sites. Changing growth rates would affect both of these parameters and might be expected to influence the need for ArfA function.

RNase III and Small Noncoding Regulatory RNAs

In *E. coli*, sRNAs control gene expression by initiating base pairing with target mRNAs. There are two general classes of sRNA. One class is mRNA gene specific and is encoded at the locus of the target gene but is transcribed from the opposite strand to generate an antisense RNA. The other class is transcribed from a separate locus outside of the target gene and is not a perfect antisense RNA. Both sRNA classes generally use helper proteins to anneal the sRNA to its target RNA. Whereas the target for a cis-encoded antisense sRNA is unique, a *trans*-encoded sRNA may have several target genes (for review, see 103). (80).

The number of *trans*-encoded sRNAs identified in *E. coli* has increased dramatically in the past few years (103). In contrast to the antisense sRNAs, which are invariably processed by RNase III, there are very few cases in which RNase III plays an unequivocal role in cleavage of paired *trans* sRNA complexes, despite the large numbers of *trans* sRNAs and their targets in *E. coli*. However, one example that demonstrates a direct effect of RNase III is the toxin/antitoxin system involving the *trans* sRNA IstR-1. IstR-1 inhibits translation of the toxin protein TisB. IstR-1 binds the *tisB* transcript upstream of the gene and reduces translation even in the absence of RNase III. When present, RNase III cleaves the paired RNAs and completely prevents TisB-induced toxicity (25, 107).

CRISPR: AN RNAi-LIKE VIRAL IMMUNITY SYSTEM IN BACTERIA THAT USES RNase III AND A *TRANS*-sRNA

Clustered regularly interspersed short palindromic repeat (CRISPR) systems have now been found in many different bacteria and function as an RNA-based defense against invading viral or plasmid DNAs (110). The genomes of these bacteria contain viral DNA segments that remain behind after a viral invasion that the bacterium survived. These viral segments are arrayed like an operon and have interspersed repeating spacer sequences (SPs) flanking each of the viral DNA segments (Figure 2). When a particular virus reinfects, long precursor RNAs, generated from the CRISPR operon, are cut into short, guide RNAs that target the viral DNA for destruction. Different CRISPR systems exist, and in most, RNA cleavage is mediated by endoribonucleases called Cas nucleases, which cleave single-strand CRISPR RNA to release the guide RNAs. However, there is one CRISPR group that uses the Cas9 nuclease plus the bacterial RNase III protein to release the guide RNAs (27). In this CRISPR system, a *trans*-encoded sRNA is expressed from outside and independently of the CRISPR sequence. This sRNA, called *tracr*-RNA, pairs with each of the repeat RNA sequences

within the precursor transcript generating a dsRNA. RNase III cleaves at the tracr-RNA-generated dsRNA repeat to release the single-strand viral RNA segment flanked by part of the repeat (27). This viral RNA is processed by Cas9 to generate the mature ~22-nt guide antiviral RNA (Figure 2) plus ~20 nts of the repeat sequence at its end (27). Cas9 uses the tracr-RNA and the mature CRISPR-guide RNA to target and destroy the homologous invading DNA (56). Given that RNase III levels vary with cell growth and environmental conditions, the CRISPR system likely varies in its ability to successfully respond to viral infections.

CRISPR and RNAi both use an RNA-mediated defense against the genomes of invaders. This similarity includes the use of RIIID/dsRBD domains to generate anti-invader guide RNAs. Such domains are evolutionarily conserved from bacteria to mammals and are present in RNase III (CRISPR) and in Drosha and Dicer proteins (RNAi).. Because of their tractability, these proteins lend themselves to many uses within the cell; here, it appears that, across evolutionary time, and through convergent pathways, similar anti-invader processes have gained hold. RNase III in concert with the CRISPR system is used as an antiviral function by bacteria, but as we see in the next section, RNase III is also used as a sensory regulator for “choosing” a developmental pathway by phage λ , when infecting *E. coli*.

RNase III IS A PHYSIOLOGICAL SENSOR FOR MODULATING DEVELOPMENT OF BACTERIOPHAGE λ

Bacteriophage λ , being a temperate phage, enters either a lytic or lysogenic developmental pathway following infection (24). Phages are replicated, cells are lysed, and phages are released during lytic development; or alternatively, the lytic cycle can be repressed and integration of the phage genome into the bacterial genome can occur, such that the phage is replicated passively by *E. coli* in the lysogenic state. How does a λ phage make this type of developmental decision?

RNase III's Crucial Role in the Decision-Making, Lifestyle Switch

Gene expression after bacteriophage λ infection depends on a cascade of transcription through terminators by a special RNA polymerase antitermination complex (39). This complex requires the phage N protein and a set of host-encoded Nus proteins. N is the master regulator in λ , and *N* is the first gene expressed in λ following infection. The 5' UTR leader, upstream of the *N* gene, contains the NUTL site to which the N and Nus proteins bind, an RNase III-sensitive hairpin (RTS), and the ribosome-binding site (SD) at the base of the hairpin (Figure 3). The RNase III hairpin interferes with ribosome binding and initiation of *N* translation (57). The N-Nus proteins bind at NUTL, then bind to the transcribing RNA polymerase that generated NUTL. This connection forms a topological RNA loop between NUTL and the transcribing polymerase (79, 111). The N-antitermination complex bound at NUTL is held next to the ribosome-binding site by the RTS structure (Figure 3a), causing translation repression of the *N* gene (113—115). Without the N-antitermination complex and N, the hairpin adjacent to the SD represses translation of N but only twofold; however, with the N complex present, there is 400-fold repression. RNase III cleavage of the hairpin eliminates all translational repression of the *N* gene. The mechanism

by which the complex binds and prevents entry of the ribosomes is not known, but it is clear that cleavage of the hairpin structure relieves the topological constraints and generates a new 5' end near the ribosome-binding site (Figure 3b), allowing high levels of N-protein synthesis (114, 115).

RNase III is a physiological sensor that determines the developmental pathway of λ infection by determining how much N protein is made. N plays a critical role in λ lytic development and N expression is controlled by the RNase III levels in the cell. Growth in rich media results in high levels of RNase III, leading to high N levels, which favors lytic development. Growth in poor media reduces RNase III levels, leading to low N levels, which favors lysogenic development (60, 114).

STRUCTURE AND CATALYTIC MECHANISMS OF RNase III

Thirty-three years after the discovery of *E. coli* RNase III, the first glimpse of the active center was provided by the structure of the *Aquifex aeolicus* RNase III (AaRNase III) enzyme (14, 121). Other structures include one of the RNA-free *Thermotoga maritima* RNase III [TmRNase III, Protein Data Bank (PDB) entry 1O0W, Joint Center for Structural Genomics] and nine of AaRNase III in complex with dsRNA (13, 41–43). The RNAs used in the crystallization experiments and observed in the structures are summarized in Figure 1b. Recently, both TmRNase III and AaRNase III have been functionally characterized using purified recombinant enzymes; the results indicate that both the protein-RNA interaction and substrate specificity of these two RNase IIIs are similar to RNase III from *E. coli* (75, 96). This is not surprising, considering the extensive conservation between bacterial RNase IIIs in both amino acid sequence and secondary structure (Figure 4a). In the following sections, residue numbers of AaRNase III will be used for consistency, unless otherwise stated.

Protein Fold, RNase III Domain (RIIID) Dimerization, and RNA-Binding Motifs

The RIIID is composed of seven α -helices, and the dsRBD exhibits the $\alpha\beta\beta\beta\alpha$ topology (Figure 4a). Whereas the RIIID fold is unique to the RNase III family (14), the dsRBD fold is also found in other dsRNA-binding proteins. In RNase III, two RIIIDs form a symmetric dimer with a ball-and-socket junction at each end of the subunit interface (14). The ball is the side chain of F41 that is located in the middle of the RNase III signature motif (Figure 4a); the socket is a cavity formed by the side chains of five residues from the partner subunit. The two junctions not only generate high-affinity binding, but also ensure accurate positioning of the two cleavage sites. These junctions have also been observed in eukaryotic RNase IIIs, including GiDicer (69) and KpDcr1 (109).

The RIIID dimerization creates the catalytic valley that accommodates dsRNA, as revealed by the crystal structure of the AaRNase III^{D44N}-Mg²⁺-RNA6 complex (D44N mutant of AaRNase III in complex with RNA6 and magnesium ion; PDB entry 2EZ6) (43). As the first catalytic complex observed for the entire family, the structure revealed a wealth of information about the mechanism of RNase III action. Various RNAs were used for cocrystallization with AaRNase III proteins, and in most cases, RNA cleavage occurred during crystallization, resulting in protein-product complexes. For example, RNA6 is the cleavage product of RNA5 (Figure 1b) (43), and once generated, two RNA6 molecules

formed a pseudoduplex RNA in the catalytic valley by annealing their 2-nt 3' overhangs. The resulting structure represents a protein-product complex rather than a protein-substrate complex. In the AaRNase III^{D44N}-Mg²⁺-RNA6 structure, however, only one Mg²⁺ is associated with the scissile bond of each RNA strand because the D44N mutation hinders the binding of the second Mg²⁺. In the structure of wild-type AaRNase III in complex with RNA9 (PDB entry 2NUG) generated as a cleavage product of RNA8 (Figure 1b), two Mg²⁺ ions are associated with each scissile bond (41).

A network of protein-RNA interactions, mediated by four RNA-binding motifs (RBMs) in the protein, is responsible for substrate specificity (Figure 4b). RBM1 and RBM2 are located within the dsRBD. RBM1 is the first α -helix of the dsRBD (Figure 4a), and it is an important specificity determinant for dsRNA binding because it forms hydrogen bonds with the O2' hydroxyl groups of RNA. RBM2 fits into the minor groove of the bound dsRNA (Figure 4b), but reorients in the opposite direction when no RNA is bound (43). RBM3 and RBM4 are located within the RIID, and when bound to RNA, position the scissile bond at the CS1, CS2 cleavage-site (Figure 4c).

Protein-Interacting Base Pairs in Hairpin Substrates and Their Sequence Specificity

The Nicholson lab has examined the hairpin RNA substrates of RNase III in great detail (see 76, 77 and references therein). Three protein-interacting boxes have been defined in the RNA substrates, termed proximal, distal (124), and middle (43) boxes (Figure 4c). RBM1 interacts with the proximal box, RBM2 interacts with the middle box, RBM3 defines the scissile bond 1 bp from the proximal box, and RBM4 interacts with the distal box (Figure 4c). Sequences paired by standard Watson-Crick pairing, especially those in the proximal and distal boxes, influence the hairpin substrate binding, positioning, and cleavage (77).

The Catalytic Valley, Processing Center, Cleavage Sites, and Mg²⁺ Ions

The catalytic valley of RNase III accommodates the dsRNA substrate, and the processing center is composed of two symmetric RNA cleavage sites (Figure 5a). The valley is negatively charged (14), but dsRNA, with its negatively charged backbone, must bind and align there for processing (43). Magnesium cations play an essential role in mitigating electrostatic repulsion between the enzyme and substrate. The dsRNA is bound outside of the catalytic valley when Mg²⁺ is absent (AaRNase III-RNA7; PDB entry 2NUE), whereas it is bound inside the valley and processed when Mg²⁺ is present (AaRNase III-Mg²⁺-RNA8; PDB entry 2NUF) (41). The other function for the Mg²⁺ ions is to coordinate a catalytically competent cleavage site assembly (Figure 5b) for two-Mg²⁺-ion catalysis (Figure 5c) (41). Each cleavage site contains a cluster of four acidic side chains that are strictly conserved among RNase III proteins (Figure 4a). There are two cleavage sites with two identical clusters of these four acidic side chains in the dimer that contribute to the net-negative charge within the catalytic valley. By converting these acidic side chains into neutral ones, as seen in the quadruple mutant protein AaRNase m^{E40Q,D44N,D107N,E110Q}, stable binding of dsRNA within the catalytic valley in the absence of Mg²⁺ can be observed (Y.-H. Liang, J.E. Tropea, B.P. Austin, D.S. Waugh, D.L. Court, X. Ji, unpublished results).

The Transition from Scissile Bond Cleavage to Product Release

The mechanism of scissile bond cleavage by RNase III can be deduced from structural data. Within the AaRNase III-Mg²⁺-RNA9 structure (PDB entry 2NUG), there are two conformations. The minor conformation (30% probability) (Figure 5b) represents the cleavage site assembly at the catalytic stage immediately after the cleavage of the phosphodiester bond and before product release, which is referred to as product-0 (Figure 5c). On the basis of this structure, a protein-substrate complex (referred to as substrate) and a protein-reaction intermediate complex (referred to as intermediate) have been confidently modeled. In the major conformation of the structure (70% probability), the 5' phosphate has moved away from the cleavage site, representing the first step toward product release after the reaction and is thus referred to as product-1. In a second structure (AaRNase III-Mg²⁺-RNA8; PDB entry 2NUF), the 3'-end product has also moved away from the cleavage site. Therefore, this structure represents a step subsequent to product release and is referred to as product-2 (Figure 5c). The two structure-based models (substrate and intermediate), in combination with the two conformations of the AaRNase III-Mg²⁺-RNA9 complex (product-0 and product-1) and the AaRNase III-Mg²⁺-RNA8 structure (product-2), predict the stepwise events during the Mg²⁺-dependent hydrolysis of the phosphodiester bond that generates the 3'-hydroxyl and 5'-phosphate ends. Here, cleavage in both strands of the dsRNA substrate creates the 2-nt 3' overhang (Figure 5a).

Modes of dsRNA Processing by RNase III

We refer to the primary mode of dsRNA processing by RNase III as the cleavage of stemloop structures within RNA transcripts, in which RNase III recognizes a stem of appropriate length, and cleaves one or both strands (23). In a second mode, RNase III can sequentially remove small RNA duplexes, ~11 bp in length, from termini of a long dsRNA by binding to the dsRNA ends, cleaving, releasing the 11 bp, and allowing a second enzyme to bind and repeat the process (23, 41, 63, 64).

HsDicer sequentially removes siRNAs from termini of long dsRNA (69, 122, 123). It recognizes the termini of dsRNA with its PAZ domain, resulting in a product cutting length that is the ~22-bp distance between the PAZ domain and the processing center in the RIIID dimer. In RNase III, it is the conserved H27 side chain (Figure 4a) that appears to dictate the length of the minimal product of sequential processing (Figure 4c). Segments exactly 11 nts in length are measured from H27 of one subunit and D44 from the partner subunit of the RIIID dimer. Mutational analysis should provide insight into the requirements of this measurement.

KpDcr1 generates siRNAs from internal regions of long dsRNA (109). In so doing, the RIIID dimers of KpDcr1 bind next to each other, and the dsRNA-product cutting length is 23 bp defined by the distance between consecutive processing centers. Using either wild-type RNase III with Mn²⁺ or the RNase III^{E38A} mutant with Mg²⁺, scientists at New England Biolabs were able to mimic KpDcr1 and produce siRNA-like duplexes 23 bp in length as a useful tool in generating reagents for RNAi experiments (117). This end-to-end type of binding by RIIID dimers was first predicted in 2001 (14) but was not observed until seven years later (41). The AaRNase III-RNA9 structure (PDB entry 2NUG) shows how the

enzyme binds end-to-end on long dsRNAs (Figure 6a) and demonstrates how the distance between consecutive processing centers defines the product length (Figure 6b).

Factors That Uncouple the RNA-Binding and Processing Activities

Three factors uncouple binding and processing: a special structural motif, such as the bulge-helix-bulge motif, embedded in dsRNA (18); defective catalytic site mutant, such as the AaRNase III^{E110K}, which corresponds to E117K in *E. coli* RNase III (26, 53, 100); and the absence of Mg²⁺ and other catalytic divalent cations (91). The structure of RNase III in complex with a dsRNA that contains the bulge-helix-bulge motif has yet to be determined. The structure of the AaRNase III^{E110K}-RNA1 complex (PDB entry 1RC7) (13) and that of the AaRNase III-RNA7 complex (PDB entry 2NUE) (41) revealed the architecture of the noncatalytic complex of RNase III. In this structure, the dsRNA is bound completely outside of the catalytic valley (Figure 7f), demonstrating how an enzyme can bind its cognate substrate without producing the expected cleavage product (83). Whereas the E110K mutation prohibits cation binding (13), the AaRNase III-RNA7 complex was obtained in the absence of divalent cations (41).

Hypothetical Pathway Leading to the Two Functional Forms of RNase III

In addition to the catalytic (Figure 7d) and noncatalytic (Figure 7f) forms of the AaRNase III^{D44N}-Mg²⁺-RNA6 complex (PDB entries 2EZ6 and 1RC7, respectively) (13, 43), structures of the AaRNase III^{E110Q}-RNA2 (Figure 7b), AaRNase III-RNA3 (Figure 7c), and AaRNase III-RNA4 (Figure 7e) complexes (PDB entries 1YZ9, 1YYW, and 1YYO, respectively) (42) revealed possible intermediate states of dsRNA binding by the enzyme. On the basis of these five forms of protein-RNA complex, together with the RNA-free conformation (PDB entry 1O0W) (Figure 7a), a hypothetical pathway that leads to the two functional (catalytic and noncatalytic) forms of RNase III in vivo is proposed.

The RNA-free RNase III dimer in conformation A (Figure 7a) first binds a dsRNA with one of the two dsRBDs. The resulting complex may have at least two possible conformations, B (Figure 7b) and E (Figure 7e), in which the dsRNA is located outside of the catalytic valley. These two conformations are expected to be interchangeable. In conformation E, the two dsRBDs pack against each other, hindering free rotation of the dsRBD-dsRNA complex around the flexible linker between the RIID and dsRBD (Figure 7e). In conformation B, however, the two dsRBDs are apart from each other, allowing free rotation of dsRBD-dsRNA around the linker (Figure 7b). The clockwise rotation of dsRBD-dsRNA leads to the catalytic form (Figure 7d) through conformation C (Figure 7c). Conformations B and C are also likely to be interchangeable. In contrast, a factor that uncouples binding and processing can further stabilize conformation E (Figure 7e), leading to the noncatalytic form of RNase III (Figure 7f) (42).

CLEAVAGE EVENTS GENERATING LONGER 3' OVERHANGS

Recently, we have observed a novel RNA cleavage event that occurs at CS3 and CS4, 1 nt outside of the typically used CS1 and CS2 sites (data presented here and in Supplemental Material; follow the Supplemental Material link from the Annual Reviews home page at

<http://www.annualreviews.org>). In two different crystallization conditions, the RNA8 substrate was processed in two different ways, resulting in RNA9 and RNA10 (Figure 1b). In the AaRNase III-Mg²⁺-RNA9 structure (PDB entry 2NUG), processing at CS1 and CS2 generated the 2-nt 3' overhang in each RNA9 stem-loop product (Figure 4c) (41). In the AaRNase III-Mg²⁺-RNA10 structure, however, cleavage also occurred at CS3 and CS4. This event removes a single cytidine residue from the 5' end of RNA8 to create RNA10, which has a 3-nt 3' overhang (Figure 8a). A second crystal structure (AaRNase III-Mg²⁺-RNA12-AMP, derived from RNA11; Figure 1b) has also demonstrated a similar 3-nt 3' overhang (see Supplemental Material).

The new cleavage events, which are also symmetric, occur at two new cleavage sites (CS3 and CS4) that flank CS1 and CS2 (Figure 8a). To distinguish the two types of RNA cleavage events, we designate the classic 2-nt 3' overhang as type I, which cleaves scissile bond 1 (between nucleotides R 0 and R-1), and the newly observed cleavage as type II, which cleaves scissile bond 2 (between nucleotides R 0 and R+1). It is the R 0 residue that becomes the free nucleotide because of the successive type I and II cleavage events (Figure 8a). Three types of overhangs can be imagined. First, the standard 2-nt overhang generated by type I cleavage; second, a 4-nt overhang generated by type II cleavage, which has not yet been seen in a structure; and a 3-nt overhang where consecutive type I and type II cleavages occur as described here (Figure 8b). The 3-nt 3' overhang might also be generated by either the combination of CS1 and CS4 or the combination of CS2 and CS3.

The two types of RNA cleavage events may be universally conserved within the entire RNase III family. With a total of four cleavage sites in the processing center of the RIID dimer, a dsRNA substrate may be processed into products containing duplexes with 3' overhangs of up to 4 nt in length. Indeed, a 16S rRNA precursor in *E. coli* was recently identified to have a 4-nt 3' overhang. This resulted from an in vivo cleavage event by RNase III during abnormally high-temperature growth conditions (4). We propose that some products generated by Dicer may have occurred using the CS3 and/or CS4 cleavage sites (10, 50). Our findings of the type II RNA cleavage event shed light on the molecular mechanism for such variations. The functional significance of these alternative cleavage sites remains to be understood. Nonetheless, we may speculate that alternative cleavage sites alter seed regions of miRNAs (50) or affect the processing and/or degradation of the RNA. Activation of this cleavage may be caused by partial melting of the duplex regions bound in or at the ends of the catalytic valley, perhaps affecting the nucleotide distance between residues H27 and cleavage site CS2 (Figure 4c).

SUMMARY

The versatility and ubiquity of RNase III and related enzymes, highlight their importance among cellular organisms and viruses alike. We have discussed the role of RNase III in gene regulation at the level of both transcription and translation, and we have examined its utility in systems that protect organisms from foreign genetic entities (i.e., viruses and plasmids). The limited production of RNase III, combined with its central role in processing core-metabolic factors, renders this protein a sensitive gauge of metabolic status, which enables fine control of a variety of genes in response to environmental stimuli. The involvement of

RNase III in processing ribosomal RNAs allows cells to rapidly produce multiple essential ribosomal components from a single RNA transcript. The ability to cleave transcripts at either the 3' or 5' UTRs of genes allows RNase III to function as both an inducer and repressor of cellular functions, and the cleavage of transcripts within coding regions enables RNase III to directly eliminate functional transcripts. Most of these activities combine the dsRNA-binding ability of the dsRBD with the catalytic cleavage ability of the RIIID of the enzyme; however, there are some instances in which RNase III exerts control over gene expression by simply binding to a transcript without cleaving it. Structural studies have illuminated the steps involved in binding and catalysis that are important for understanding the biological activities of RNase III, and they have revealed some interesting new findings. RNase III displays an alternative mode of action that leads to the heterogeneity of cleavage products, resulting in 2-, 3-, and 4-nt 3' overhangs at the cleavage site. These newly discovered products may have important biological consequences that will be revealed in the years to come.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Summary Points

1. RNase III is regulated at posttranscriptional and posttranslational levels and by growth rate and other environmental factors.
2. RNase III is a global regulator of gene expression in *E. coli*.
3. RNase III controls gene expression by cleaving double-stranded structures within coding sequences, the 5' UTR, and the 3' UTR.
4. RNase III controls gene expression by binding to double-stranded structures within the 5' UTR and the 3' UTR.
5. RNase III exhibits two distinct modes of dsRNA binding.
6. RNase III generates 3' overhangs of 2, 3, or 4 nts in the processing center.

Figure 1. Representative RNase III proteins and RNA oligos used and/or observed in the structures of *Aquifex aeolicus* (Aa) RNase III-RNA complexes. (a) *Homo sapiens* Dicer (1,922 amino acid residues, UniProtKB Q9UPY3), *Giardia intestinalis* Dicer (754 residues, UniProtKB A8BQJ3), *H. sapiens* Drosha (1,374 residues, UniProtKB Q9NRR4), *Trypanosoma brucei* mRPN1 (486 residues, UniProtKB Q384H9), *Kluyveromyces polysporus* Dcr1 (558 residues, UniProtKB A7TR32), *Candida albicans* Dcr1 (611 residues, UniProtKB Q5A694), *Saccharomyces cerevisiae* Rnt1p (471 residues, UniProtKB Q02555), *Escherichia coli*

RNase III (226 residues, UniProtKB P0A7Y0), and *Bacillus subtilis* Mini-III (143 residues, UniProtKB O31418). The scale on top indicates the lengths of polypeptide chains. Domains are indicated by colored boxes. The red box in the RNase III domain indicates the RNase III signature motif. (b) Small synthetic RNAs used in this and previous structural analyses as referenced, namely RNA1 (13); RNA2, RNA3, and RNA4 (42); RNA5 and RNA6 (43); RNA7, RNA8, and RNA9 (41); RNA10, RNA11, and RNA12 (this work). Red arrowheads indicate sites where cleavage occurred during crystallization.

Figure 2.

The type II CRISPR (clustered regularly interspersed short palindromic repeats) pathway is outlined from the CRISPR operonic DNA to its guide RNA product. Tracr-RNA is a *trans*-encoded sRNA transcribed independently of the CRISPR operon. This CRISPR system uses RNase III to process the preCRISPR RNA and was first found in *Streptococcus pyogenes*.

Figure 3.

The RNA polymerase/λ N transcription antitermination complex transcribing the N gene (a) before or (b) after cleavage at the RNaseIII-sensitive hairpin (RTS). The RNA transcript in black is transcribed from the *P_L* promoter and contains the N/Nus factor-binding site (NUTL), the RNase III sensitive hairpin (RTS), the ribosome-binding site (SD). The scissors represent cleavage by RNase III. Ribosome subunits 30S and 50S are indicated.

Figure 4. Sequence and structure of bacterial RNase IIIs. (a) Structure-based sequence alignment of AaRNase III (UniProtKB O67082), TmRNase III (UniProtKB Q9X0I6), and EcRNase III (UniProtKB P0A7Y0). The RNase III domain (RIIID) and double-stranded RNA-binding domain (dsRBD) are boxed. Secondary structural elements are shaded (*helices in gray and strands in yellow*). Underlined in red are the RNase III signature motif and the linker between RIIID and dsRBD. RNA-binding motifs (RBMs) 1–4 are underlined in blue. (b) The location of RBMs 1–4 in the AaRNase III-Mg²⁺-RNA9 structure (PDB entry 2NUG).

One subunit of AaRNase III (*backbone in cyan*) is shown with the RNA pseudoduplex (*molecular surface in light gray*). The cleavage site is indicated by the position of two Mg^{2+} ions (*spheres in black*). Highlighted are the RBMs *blue* and the linker between the RIIID and dsRBD (*red*). (c) Schematic illustration of AaRNase III-RNA interactions as observed in the AaRNase III- Mg^{2+} -RNA9 structure (PDB entry 2NUG), showing two subunits of AaRNase III (blue and red), each containing a RIIID (RBMs 3–4) and a dsRBD (RBMs 1–2). The nucleotide residues in the dsRNA are shown as small rectangle boxes, and the two cleavage sites (CSs 1–2) are indicated with arrowheads. The conserved amino acid residue H27 from subunit 1 interacts with the 11th nucleotide residue from CS2, dictating the typical product length of 11 nts when bacterial RNase III processes long dsRNA. The proximal, middle, and distal boxes are outlined and indicated with P, M, and D. The nucleotide residue in the cleavage site is numbered R 0; the rest are numbered according to the polarity of the RNA strand.

Protein fold, RNase III-domain (RIIID) dimerization, catalytic assembly, and cleavage mechanism. (a) Schematic view of the AaRNase III-Mg²⁺-RNA9 structure (PDB entry 2NUG). The two RIIIDs are shown as molecular surfaces, the two double-stranded RNA-binding domains (dsRBDs) are illustrated as ribbon diagrams (*α -helices as spirals, β -strands as arrows, and loops as pipes*), and the two subunits are colored in cyan and orange. The Mg²⁺ ions are shown as black spheres, and the two RNA strands are shown as tube-and-stick models in blue and red. (b) In the AaRNase III-Mg²⁺-RNA9 structure (PDB entry 2NUG), each cleavage site assembly is composed of the 3' hydroxyl and 5' phosphoryl products, two Mg²⁺ ions, three water molecules, and four catalytic side chains; the assembly exhibits

alternate conformations at the ratio of 0.3/0.7 (41). In the minor conformation (30% probability), the distance between the 3' hydroxyl and the 5' phosphorus is 3.0 Å, shorter than the sum of the van der Waals radii of phosphorus (1.9 Å) and oxygen (1.4 Å), thus representing the cleavage site arrangement immediately after RNA cleavage. The amino acid and nucleotide residues are shown as stick models and Mg^{2+} ions, and water oxygens are shown as spheres in atomic color scheme (C, *gray*; N, *blue*; O, *red*; P, *orange*; Mg, *black*). Metal coordination bonds are indicated by solid lines, and hydrogen bonds are indicated by dashed lines. The nucleotide residue in the middle is numbered R 0, and the rest are numbered according to the polarity of the RNA strand. (c) The stepwise model for phosphoryl transfer and product release. The molecular models for the protein-substrate complex (substrate) and protein-reaction intermediate complex (intermediate) are constructed on the basis of the cleavage-site arrangement immediately after the RNA cleavage (product-0; minor conformation in PDB entry 2NUG), whereas the release of the 5'-end product (product-1) followed by the 3'-end product (product-2) from the cleavage site is represented by the major conformation in the AaRNase III- Mg^{2+} -RNA9 structure (PDB entry 2NUG) and the AaRNase III- Mg^{2+} -RNA8 structure (PDB entry 2NUF). Panel c was originally published in Reference 41.

Figure 6.

Cooperative binding mode of AaRNase III to dsRNA. (a) The cooperative binding mode of AaRNase III to dsRNA as observed in the AaRNase III-Mg²⁺-RNA9 structure (PDB entry 2NUG). The AaRNase III is illustrated as a graphic model with the two subunits colored in pale cyan and light orange, and each subunit is outlined by a transparent molecular surface. The pseudoduplex RNA is illustrated as a tube-and-stick model in gray with the product siRNA highlighted in blue and red. (b) Schematic illustration showing how the siRNA length is determined by the distance between the processing centers of adjacent RIIID dimers.

Figure 7.

Hypothetical pathways leading to two functional forms of RNase III. Six distinct conformational states are represented by (a) RNA-free TmRNase III (PDB entry 1O0W), (b) AaRNase III^{E110Q}-RNA2 (PDB entry 1YZ9), (c) AaRNase III-RNA3 (PDB entry 1YYW), (d) AaRNase III^{D44N}-Mg²⁺-RNA6 (PDB entry 2EZ6), (e) AaRNase III-RNA4 (PDB entry 1YYO), and (f) AaRNase III^{E110K}-RNA1 (PDB entry 1RC7). The RNase III domain (RIID) dimer is illustrated as a molecular surface with positive and negative potentials indicated by blue and red, respectively; the double-stranded RNA-binding domains (dsRBDs) are shown as backbone worms in white; and the dsRNA is represented as stick models in atomic color scheme (*carbon in white, nitrogen in blue, oxygen in red, phosphorous in yellow*). The direction of predicted rotation of the dsRBD-dsRNA complex, enabled by the flexible linker between the RIID and dsRBD, is indicated with arrowheads on the circles. The orientation of the RIID moiety was kept constant. Panels a, b, c, e, and f were originally published in Reference 42, whereas panel d is new.

Figure 8. The type II RNA cleavage event. (a) The RNase III-dsRNA interactions observed in the AaRNase III-Mg²⁺-RNA10-CMP and AaRNase III-Mg²⁺-RNA12-AMP structures are schematically illustrated. The dimeric protein is shown as two rectangles and labeled as subunit 1 and subunit 2, and the four RNA-binding motifs (RBMs) as ellipses. The cleavage sites (CSs 1–4) are indicated with arrowheads, and the proximal, middle, and distal boxes are outlined and indicated with one letter abbreviations. For each RNA strand, the nucleotide residue between the two cleavage sites is numbered R 0, and the rest are numbered according to the polarity of the RNA strand. (b) The creation of a 2-, 3-, or 4-nt 3' overhangs by type I, type I followed by type II, or type II cleavage events, respectively. The type I (CS1, CS2) event is commonly seen in vivo and in structures. The type I followed by typeII event (CS1, CS2; CS3, CS4) is first described here in the two structures above yielding 3-nt overhangs. The type II only event (CS3, CS4) has only been seen in vivo and yields 4-nt overhangs (4). The other type I followed by type II events (CS1, CS4 and CS2, CS3) are hypothetical and have not been seen.