Ribonuclease III Processing of Coaxially Stacked RNA Helices*

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The RNase III family of endoribonuclease participates in maturation and decay of cellular and viral transcripts by processing of double-stranded RNA. RNase III degradation is inherent to most antisense RNA-regulated gene systems in Escherichia coli. In the hok/sok system from plasmid R1, Sok antisense RNA targets the hok mRNA for RNase III-mediated degradation. An intermediate in the pairing reaction between Sok RNA and hok mRNA forms a three-way junction. A complex between a chimeric antisense RNA and hok mRNA that mimics the three-way junction was cleaved by RNase III both in vivo and in vitro. Footprinting using E117A RNase III binding to partially complementary RNAs showed protection of the 13 base pairs of interstrand duplex and of the bottom part of the transcriptional terminator hairpin of the antisense RNA. This suggests that the 13 base pairs of RNA duplex are coaxially stacked on the antisense RNA terminator stem-loop and that each stem forms a monomer half-site, allowing symmetrical binding of the RNase III dimer. This processing scheme shows an unanticipated diversity in RNase III substrates and may have a more general implication for RNA metabolism.

RNase III of Escherichia coli (1) belongs to a family of double strand-specific endoribonucleases (2, 3), which have retained interspecies substrate cleavage specificity (4). This family of enzymes comprises both prokaryotic and eukaryotic members whose primary function is processing of ribosomal RNA precursors (4–6). However, substrates also include several mRNAs from E. coli, bacteriophages λ and T7 (2, 3), U5 small nuclear RNA (7), U2 small nuclear RNA (8), and a large subset of small nuclear RNA precursors of Saccharomyces cerevisiae (9, 10). Substrate recognition requires two helical turns of an A-form RNA helix, and processing occurs with a consensus 2-nucleotide, 3′-recessive, staggered cut creating 5′-phosphate and 3′-hydroxyl termini (11, 12). The E. coli RNase III holozyme is a homodimer of 52 kDa that binds dsRNA in a quasi-symmetrical fashion concesntral of the scissile phosphodiester bonds (13). RNase III binding is dependent on a conserved dsRNA-binding motif present in the C-terminal end that forms an α-β-β-α fold (14). Binding of dsRNA is believed to occur primarily via non-electrostatic interactions with a helical arrangement of 2′-hydroxyl groups in the minor groove as described for the dsRNA-binding motif of the mammalian dsRNA-activated protein kinase, PKR (15). In addition, a lysine residue may be required for dsRNA binding, similar to what has been observed for the staufen protein from Drosophila (16) and PKR (17–19). Binding of the dsRBD of RNA-binding protein A from Xenopus laevis to dsRNA has shown that two regions of the dsRBD contact successive minor grooves on the same face of the double-stranded RNA helix, whereas a third region contacts the spanning major groove (20). However, the recent finding of binding sequence anti-determinants in dsRNA that abolish RNase III cleavage in vitro (21) emphasizes the subtleties of substrate recognition by RNase III and may explain why certain dsRNA sequences like the human immunodeficiency virus type 1 TAR RNA hairpin are inherently refractory to processing (4).

Complete or partial duplexes of ≥20 bp formed between antisense RNAs and their target RNAs are efficiently processed by RNase III (22–26). The hok (host cell killing) mRNA from the hok/sok (suppression of killing) system of plasmid R1 (27) exists in three forms with alternative configurations and translational capacities (28). Sok antisense RNA consists of an 11-nucleotide 5′-single-stranded tail that is responsible for the initial interaction with hok mRNA and a hairpin that functions as a Rho-independent transcriptional terminator (Fig. 1A). Sok RNA represses hok translation by forming a 63-bp duplex with hok mRNA (Fig. 1C). The duplex is cleaved by RNase III in vivo and in vitro (26). Since formation of RNA/RNA binding intermediates could be faster than the formation of a full duplex between Sok RNA and hok mRNA, inactivation of the target RNA could occur prior to complete duplex formation, as in the cases of several other antisense RNA-regulated gene systems (29). To test this, we constructed a chimeric antisense RNA (CA-RNA) consisting of the 13-nucleotide 5′-tail of Sok fused to the terminator hairpin of PndB, an antisense RNA from plasmid R483 that is homologous to Sok (Fig. 1B). CA-RNA forms a 13-base pair duplex with hok mRNA, thereby resulting in the generation of a three-way junction (Fig. 1D, left panel). This complex mimics the naturally occurring binding intermediate between hok mRNA and Sok antisense RNA (Fig. 1D, right panel). Surprisingly, CA-RNA was able to inhibit hok expression in vivo,2 thus questioning the actual mechanism of hok target inactivation by Sok RNA.

Here, we have compared the effect of Sok RNA and CA-RNA on hok mRNA metabolism in rnc and rnc strains. Complexes between antisense and target RNAs that correspond to the RNA complexes observed in vivo were assayed for RNase III binding and processing in vitro. Intriguingly, all data support a model in which RNase III recognizes and cleaves coaxially stacked RNA helices.

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¶ The abbreviations used are: dsRNA, double-stranded RNA; dsRBD, double-stranded RNA-binding domain; bp, base pair(s); CA-RNA, chimeric antisense RNA; TWJ, three-way junction.
Novel RNase III Substrate Specificity

Experimental Procedures

Materials—Uniformly labeled RNAs (α-[^32]P)CTP or α-[^32]H)CTP) were synthesized using T7 RNA polymerase (Promega) and polymerase chain reaction-generated templates (30). 5′-[^32]P-Labeled RNAs were prepared with alkaline phosphatase (Roche Molecular Biochemicals), T4 kinase (Roche Molecular Biochemicals), and (α-[^32]P)ATP using standard techniques (31). RNAs were purified as described (30).

Construction of Plasmids—The hok/lock homologous antisense RNAs were cloned into an expression vector (pBR322) that carries a T7 promoter. These constructs were then inserted into T7-AN1, a plasmid containing a T7 promoter and a translational start codon (amino acid 1) that is followed by an N-terminal His6 tag, into strains W3110 and HT115, which were transformed as described (33). Eluted protein was dialyzed against 1X TMK-glutamate buffer supplemented with 1 mM dithiothreitol and 50 mM KCl. The E117A amino acid substitution was introduced by double polymerase chain reaction using the above primers and rnc-E117A-1 (5′-CGGACACCGTCGCAATTGGA-3′) and rnc-E117A-2 (5′-CCATTTAATCTGTCGACGGAATGGTCCG-3′), generating pTF601. LacI was expressed from the co-resident plasmid pMS421, which carries the lacI gene, and the resulting strain was transformed with the plasmid pTF601. The pTF322 to pTF324 plasmid series was constructed by polymerase chain reaction using the above primers and pBR322 antisense RNA-donating plasmid, respectively. 10 mIU tetacycline was added to the HT115 strain.

Lead(II) Acetate Probing—5′-End-labeled CA-RNA or hok38 RNA was incubated either alone or in complex with an excess (1 pmol) of partner RNA in a buffer (50 mM Hepes-KOH (pH 7.5), 10 mM MgCl2, and 50 mM KCl) supplemented with 5 μg of tRNA in a total reaction volume of 10 μl. Lead(II) acetate (2.5 μl) dissolved in H2O immediately prior to use was added to a final concentration of 0, 5, or 10 mM and incubated for 5 min at room temperature. Reactions were quenched by addition of EDTA to a final concentration of 40 mM. The RNA was precipitated, washed twice, resuspended in formamide dye, and subsequently resolved on 15% polyacrylamide gels containing 7 M urea and 1X Tris borate/EDTA.

RNA Cleavage Assay—5′-nt RNA was incubated in the presence or absence of an 10-fold excess of various complementary partner RNAs in 1X poly(I)/poly(C) (Promega) buffer. 0.1 unit of calf intestine RNase A (Promega) was added to each reaction, and 32P-labeled antisense RNA and CA-RNA were incubated together to form preformed complexes. The E117A amino acid substitution was introduced by double polymerase chain reaction using the above primers and rnc-E117A-1 (5′-CGGACACCGTCGCAATTGGA-3′) and rnc-E117A-2 (5′-CCATTTAATCTGTCGACGGAATGGTCCG-3′), generating pTF601. The E117A amino acid substitution was introduced by double polymerase chain reaction using the above primers and rnc-E117A-1 (5′-CGGACACCGTCGCAATTGGA-3′) and rnc-E117A-2 (5′-CCATTTAATCTGTCGACGGAATGGTCCG-3′), generating pTF601. LacI was expressed from the co-resident plasmid pMS421, which carries the lacI gene, and the resulting strain was transformed with the plasmid pTF601. The pTF322 to pTF324 plasmid series was constructed by polymerase chain reaction using the above primers and pBR322 antisense RNA-donating plasmid, respectively. 10 mIU tetacycline was added to the HT115 strain.

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RNA Cleavage Assay—5′-nt RNA was incubated in the presence or absence of an 10-fold excess of various complementary partner RNAs in 1X poly(I)/poly(C) buffer. 0.1 unit of RNase III dimer was added, and reactions were quenched by addition of EDTA to a final concentration of 40 mM. The RNA was precipitated, washed twice, resuspended in formamide dye, and subsequently resolved on 15% polyacrylamide gels containing 7 M urea and 1X Tris borate/EDTA.

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RESULTS

In Vivo RNase III Processing of hok mRNA—To test the effect of natural and artificial antisense RNAs on the level of hok mRNA in vivo, a hok/sok system with abolished Sok RNA expression was constructed and cloned into a low-copy number plasmid containing the hok gene. Northern transfer analysis of hok mRNAs in W3110 (rnc+; left panel) and HT115 (rnc−; right panel). The hok mRNAs were expressed from a mini-R1 plasmid carrying either hok / sok+ (pTF820) (lane 1) or hok / sok− (pTF821) (lanes 2–6) gene systems. The hok− and sok− genotypes denote an amber mutation in the hok gene preventing toxin expression and a mutation in the sok promoter that abolishes Sok RNA expression, respectively (32). Lane 3, the pBR322 control plasmid; lanes 4–6, wild-type Sok RNA (pTF322) and CA-RNA (pTF323) and PndB (pTF324) antisense RNAs produced in trans from pBR322-derived plasmids, respectively. Translationally inactive hok mRNA-1 and -2 as well as the active truncated (Tr.) hok mRNA are indicated. hok mRNA-2 is not produced in the rnc− strain.

In Vitro RNase III Processing of the TWJ—Different antisense RNA/target RNA combinations were tested for their ability to function as RNase III-processing signals in vitro using purified histidine-tagged RNase III (Fig. 4). The cleavage reactions were performed at high stringency (i.e. at a high concentration of monovalent salt). As expected, truncated hok mRNA alone was not affected by RNase III (Fig. 4A, left panel), consistent with the extraordinary stability of this mRNA in vivo (28). Addition of Sok RNA resulted in a major cleavage product in addition to multiple consecutive cleavage fragments (MCF; Fig. 4A, middle panel). In contrast, addition of CA-RNA to truncated hok mRNA yielded two well defined cleavage products, indicative of a single specific cleavage site. Consistently, the longest cleavage fragments in the two reactions were of similar sizes (Fig. 4A).

We also examined RNase III cleavage of labeled wild-type Sok RNA (Fig. 4B) and CA-RNA (Fig. 4C). Alone, both RNAs were resistant to RNase III processing. The lack of cleavage is consistent with the presence of upper stem helix irregularities that may act to protect the RNAs from RNase III cleavage. Such stem irregularities are known to prevent RNase III cleavage (35). Addition of truncated hok mRNA resulted in multiple cleavages of Sok RNA (Fig. 4B, middle panel) and a single cleavage of CA-RNA (Fig. 4C, middle panel), consistent with the cleavage pattern observed for hok mRNA in Fig. 4A. These results show that all RNase III cleavages described here reflect coordinated double-strand scissions. Furthermore, primer-extension analysis showed that all double-strand cleavages occurred at the phosphodiester bond between nucleotides +13 and +14 of the antisense 5′-tail and the corresponding phosphodiester bond in hok mRNA, resulting in a 2-nucleotide 3′-overhang according to consensus RNase III processing (data not shown). This cleavage site is designated position 13/11 below.

As implied by the in vivo and in vitro RNase III processing experiments, a partial duplex between the CA-RNA antisense RNA and the hok mRNA seems to be sufficient for RNase III processing. A 14-nucleotide fragment (hok13) of hok mRNA sequence that is complementary to the 13-nucleotide 5′-tail of
Sok RNA and CA-RNA was synthesized (the fragment carried an additional 5'-G for efficient transcription by T7 RNA polymerase). Addition of hok13 to Sok RNA or CA-RNA resulted in single specific RNase III cleavages (Fig. 4, B and C, right panels) at the same processing site at position 13/11 described above (data not shown). These observations are consistent with the in vivo processing data and confirm that a partial antisense RNA/target RNA duplex comprising 13 bp of interstrand pairing is adequate for RNase III processing.

To test if all 13 bp of the duplex were required for processing, we examined 3'-end-shortened hok mRNA fragments of 7 (hok7) and 10 (hok10) nucleotides for RNase III-mediated CA-RNA cleavage (Fig. 4D), hok7 failed to sustain cleavage (Fig. 4D, left panel), whereas partial cleavage was observed with hok10 (middle panel). Thus, all 13 base pairs are required for optimal enzyme binding or processing. In addition, the cleavage observed with hok10 occurred at the site at position 13/11 described above, implying that binding and/or cleavage is fixed at a unique position and does not change with the 5'-end border of the partial duplex (data not shown).

**Cleavage Kinetics and Substrate Affinity**—The kinetic parameters for RNase III processing of Sok RNA in duplex with hok13 and CA-RNA in duplexes with hok13 and hok38 are shown in Table I. The $k_{cat}$ and $K_m$ values for RNase III processing of all three complexes are similar to the values calculated for the R1.1 substrate from bacteriophage T7 using wild-type RNase III (33). In addition, the formation of a TWJ in the CA-RNA:hok38 complex does not impair affinity or cleavage rate compared with the Sok RNA:hok13 and CA-RNA:hok13 complexes. Thus, the in vitro kinetic parameters for RNase III processing of the partial duplexes clearly support the ability of this substrate to compete for RNase III binding and cleavage in vivo.

The **Transcriptional Terminator Stem-Loop Structure** Is **Pre-requisite for RNase III Cleavage of hok13**—Complexes formed between uniformly $^{32}$P-labeled hok13 and Sok RNA, CA-RNA,

**Fig. 3.** Demonstration that CA-RNA and the minimal target RNA form at the three-way junction. Shown are the results from lead(II) acetate probing of the complex formed between CA-RNA and hok38 RNA. A, 50 fmol of $^{32}$P-5'-end-labeled CA-RNA (left panel) or hok38 (right panel) was incubated either alone or in complex with an excess (1 pmol) of unlabeled hok38 or CA-RNA, respectively. Lead(II) acetate was added at the concentrations indicated. RNase T1 cleavage reactions (T1) were carried out as (36) on native RNA. L denotes the alkaline ladders. The loops of CA-RNA and hok38 and the single-stranded 5'-tail of CA-RNA are indicated. The bulged-out A$^C$ of hok38 is indicated to aid cleavage site localization. B, shown is the structure of the complex formed between CA-RNA and hok38 based on the lead(II) acetate probing results in A. The 13 nucleotides of the 5'-tail of CA-RNA that are complementary to the target in hok38 are shown in boldface, and the RNA stem-loop structure derived from the PndB antisense RNA in lightface. Lead(II) acetate cleavages of labeled CA-RNA and hok38 that are unaffected by complex formation are indicated by asterisks, whereas cleavage sites repressed upon complex formation are indicated by boldface lines. RNase T1 cleavage sites are indicated with thin arrows.
or Sok13, which corresponds to the 13 nucleotides of the 5’-tail sequence of Sok RNA (and CA-RNA) were examined for accurate RNase III cleavage (Fig. 5). Binding of either Sok RNA or CA-RNA resulted in RNase III cleavage at the specific site in hok13. In contrast, RNase III failed to perform accurate processing of the hok13-Sok13 complex. Hence, the base pair interstrand RNA duplex is not a substrate for RNase III. These results show that the transcriptional terminator hairpins of Sok RNA and CA-RNA are necessary for RNase III processing. The cleavage sites at position 13/11 suggest that the 13 bp of antisense RNA/target RNA duplexes and the bottom stem of the antisense RNA transcriptional terminator hairpins each constitute RNase III monomer half-sites.

**RNA III Binding to Coaxially Stacked RNA Helices—**

RNase III binding was examined using an RNase III derivative carrying an E117A amino acid substitution. This protein shows binding properties identical to those of the wild-type enzyme, but is impared in substrate cleavage (13). **Al**.End-labeled CA-RNA was incubated either alone or with hok13 in the presence or absence of E117A RNase III (Fig. 6A). As the substrates tested contained almost exclusively double-stranded or stacked nucleotides, we used cobra venom nuclease to monitor substrate binding (Fig. 6B). At both concentrations of E117A RNase III, the unpaired CA-RNA was not protected from cobra venom nuclease cleavages. Adding the hok13 fragment resulted in E117A RNase III-mediated protection of the 5’-tail and bottom hairpin at both the 5’- and 3’-sides. These data are consistent with a symmetrical binding of E117A RNase III coaxially stacked helices that comprise the bottom part of the CA-RNA transcriptional terminator hairpin stacked on the 13 bp of the interstrand CA-RNA/hok13 duplex. A structural model showing the protection of the CA-RNA/hok13 complex by E117A RNase III is presented in Fig. 6B. Protection extends roughly 10–12 bp on each side of the cleavage sites, which are located in the hinge region of the stacked helices (Fig. 6A).

**DISCUSSION**

Many antisense RNAs target their complementary transcripts for RNase III-mediated degradation. Although the antisense RNAs of the Sok RNA family can form complete duplexes with their target RNAs, our data show that a pairing intermediate of 13 base pairs that forms a TWJ is sufficient for RNase III-mediated degradation in vivo. Intriguingly, substrates corresponding to the transcripts examined in vivo were cleaved by purified RNase III at a single specific site in both strands of the antisense RNA/target RNA duplexes. Our data support a minimal substrate that consists of the antisense RNA transcriptional terminator hairpin and the 13 nucleotides of the 5’-tail engaged in RNA interstrand pairing. However, RNase III processing normally requires at least two helical turns of RNA duplex (2, 3). Indeed, the 13 bp of duplex are necessary, yet insufficient for RNase III cleavage (Fig. 4). E117A RNase III protein footprinting supports enzyme binding at the 13 bp of interstrand duplex and at the bottom 10–12 bp.

**TABLE I**

| RNA complex         | \(k_{cat}\) | \(K_m\) | \(k_{cat}/K_m\) |
|---------------------|------------|--------|----------------|
| Sok RNA · hok13     | 6.4        | 260    | 2.5 \times 10^2 |
| CA-RNA · hok13      | 6.6        | 235    | 2.8 \times 10^2 |
| CA-RNA · hok38      | 6.4        | 245    | 2.6 \times 10^2 |

**FIG. 4. In vitro RNase III processing of the three-way junction and of coaxially stacked RNA helices**. Uniformly 32P-labeled hok mRNA (A), Sok RNA (B), and CA-RNA (C and D) were preincubated either alone or with a 10-fold excess of \(3^\text{H}\)-labeled partner RNA as indicated for 30 min before addition of N-terminal His\(_6\)-tagged RNase III at 37 °C (A–C) or 25 °C (D). Samples were withdrawn at the time points indicated. Unsealed RNA (UC), the 5’- and 3’-end cleavage products, and the multiple cleavage fragments (MCF) resulting from consecutive RNase III processings are indicated by arrowheads.

**FIG. 5. In vitro RNase III cleavage of uniformly 32P-labeled hok13 transcripts**. The assays were conducted as described in the legend to Fig. 3 at 37 °C. The alkali ladder (L) was made on a 32P-5’-end-labeled hok13 fragment as described (36). Uncleared RNA (UC) and the 5’- and 3’-cleavage products corresponding to 3 and 11 nucleotides, respectively, are indicated.
Novel RNase III Substrate Specificity

Low binding affinity for such a monomer/half-site interaction is expected to increase the stringency in substrate binding and to prevent titration of RNase III by dsRNA elements that are not RNase III substrates. The $K_m$ and $k_{cat}$ values for CA-RNA in partial duplex with hok13 and hok38 and for Sok RNA in complex with hok13 are similar to the values previously reported for RNase III processing (33) and concur with the observation that the CA-RNA/hok mRNA substrate is efficiently processed in vivo. Thus, formation of the TWJ in the CA-RNA/hok mRNA complex (Figs. 1D and 3) does not impede RNase III processing (Figs. 2 and 4 and Table 1), which could suggest that the enzyme does not form a tightly closed complex surrounding the cleavage site since the extra RNA stem is accommodated by the catalytic site without affecting $k_{cat}$.

Several RNase III substrates have structural irregularities (i.e. bulges, mismatches, and internal loops) (2) around the scissile bonds, including the Sok RNA/hok13 and CA-RNA/hok13 complexes and the R1.1 substrate of bacteriophage T7. However, data obtained with the native R1.1 substrate of bacteriophage T7 and an engineered version with perfect helicity showed similar equilibrium dissociation constants, suggesting that little or no discrimination on substrate binding is conferred by the catalytic site (13). Instead, the recent finding of RNase III binding anti-determinants implies that the stringency of processing is on the level of enzyme binding exclusively (21). Consistent with the efficient processing, none of the substrates tested here convey the RNase III binding anti-determinants. Alignment of RNase III-processing signals from E. coli has identified a preference for a C-G base pair at position +6/+4 relative to the scissile bonds (21, 37). Interestingly, the CA-RNA/hok mRNA complex contains one C-G base pair in the tail/target duplex and one C-G base pair in the terminator hairpin of CA-RNA located symmetrically (disregarding the bulged-out U) at position +6/+4 (Fig. 6B). The crystal structure of the dsRBD of the dsRNA-binding protein A of X. laevis in complex with dsRNA revealed a single sequence-specific contact between a backbone carbonyl group and the exocyclic amine of G in the minor groove of a C-G base pair (20). Thus, a similar interaction could be important for RNase III binding of its substrates and may in some cases specify the cleavage site location. This is supported by the fixed cleavage site at position 13/11 observed here for RNase III processing of the CA-RNA/hok10 complex, for which the end of the interstrand duplex has been juxtaposed, compared with the CA-RNA/hok mRNA and CA-RNA/hok13 complexes.

The fact that the family of RNase III enzymes shows at least partial interspecies substrate specificity (4) suggests that coaxially stacked helices could function as processing signals for RNase III enzymes in both prokaryotic and eukaryotic cells. Recently, an RNase III-processing signal composed of noncontiguous helices was suggested for the small nuclear R40 precursor of S. cerevisiae (10). However, for this substrate, the junction of the RNA helices is positioned 6–8 base pairs from the scissile phosphodiester bonds, most likely displaced from the catalytic site.

Intriguingly, binding of coaxially stacked dsRNA by two copies of the dsRBD of PKK may apply to a diverse set of proteins with unrelated function. In a recent experiment, the dsRBD of PKK was shown to bind in vitro selected RNA with noncontiguous and most likely coaxially stacked RNA helices (38). Thus, it is conceivable that enzymes like PKK and ADAR (adenine deaminase that acts on RNA) that carry multiple copies of the dsRBD can bind coaxially stacked RNA stems in a fashion similar to RNase III and thereby trigger such diverse cellular responses as apoptosis, the interferon-induced viral response (39, 40), or RNA degradation (41).
The processing of coaxially stacked RNA helices reported here shows that RNase III substrates are more versatile than previously assumed. This novel substrate specificity could have a more general implication on the metabolism of diverse RNAs.

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