Absence of RNase III alters the pathway by which RNAI, the antisense inhibitor of ColE1 replication, decays

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RNAI is a short RNA, 108 nt in length, which regulates the replication of the plasmid ColE1. RNAI turns over rapidly, enabling plasmid replication rate to respond quickly to changes in plasmid copy number. Because RNAI is produced in abundance, it is easily extracted and turns over quickly, it has been used as a model for mRNA in studying RNA decay pathways. The enzymes polynucleotide phosphorylase, poly(A) polymerase and RNase E have been demonstrated to have roles in both messenger and RNAI decay; it is reported here that these enzymes can work independently of one another to facilitate RNAI decay. The roles in RNAI decay of two further enzymes which facilitate mRNA decay, the exonuclease RNase II and the endonuclease RNase III, are also examined. RNase II does not appear to accelerate RNAI decay but it is found that, in the absence of RNase III, polyadenylated RNAI, unprocessed by RNase E, accumulates. It is also shown that RNase III can cut RNAI near nt 82 or 98 in vitro. An RNAI fragment corresponding to the longer of these can be found in extracts of an rnc M pcnB strain (which produces RNase III) but not of an rnc pcnB strain, suggesting that RNAI may be a substrate for RNase III in vivo. A possible pathway for the early steps in RNAI decay which incorporates this information is suggested.

Keywords: Escherichia coli, plasmid replication, RNA degradation, ribonuclease III, RNAI

INTRODUCTION

ColE1 regulates its copy number with a short RNA counter-transcript, RNAI. RNAI can hybridize to the complementary portion of a longer RNA, RNAII, and thus prevent its maturation into the primer used to initiate ColE1 replication. RNAI is synthesized constitutively from a strong promoter and its steady-state level is high relative to that of RNAII. However, it turns over rapidly. As a result, its concentration alters quickly in response to a change in plasmid copy number and replication is immediately inhibited or stimulated as appropriate (for reviews see Cesareni et al., 1991; Wagner & Simons, 1994).

RNAI breakdown is rapid, and, like that of mRNAs (reviewed by Nierlich & Murakawa, 1996; Kushner, 1996), is accomplished by a combination of endonucleases and exonucleases. For these reasons, it has been studied as a model for mRNA decay. Major roles in RNAI decay have been demonstrated for a number of enzymes. These enzymes are listed in Table 1. RNase E (Tomcsányi & Apirion, 1985) is an essential enzyme with endonucleolytic activity important in messenger decay (Kuwano et al., 1977) and rRNA processing (Ghora & Apirion, 1978). Lin-Chao & Cohen (1991) showed that it has a major role in RNAI decay. Polynucleotide phosphorylase (PNPase) is one of the two exonucleases implicated in mRNA degradation (Donovan & Kushner, 1986). Xu & Cohen (1995) reported on its role in RNAI decay. Poly(A) polymerase (PAP I), discovered as a consequence of its role in plasmid copy number maintenance (Lopilato et al.,...
Table 1. RNA decay enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Gene</th>
<th>Mutation</th>
<th>Enzyme activity</th>
<th>Substrate</th>
</tr>
</thead>
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<tr>
<td>Ribonuclease E (RNase E)</td>
<td>rne</td>
<td>-1 (Ts)</td>
<td>Endonuclease (single strand)</td>
<td>mRNA, RNAI</td>
</tr>
<tr>
<td>Ribonuclease E (RNase E)</td>
<td>rne</td>
<td>-3071 (Ts)</td>
<td>Exonuclease (3' → 5')</td>
<td>mRNA, RNAI</td>
</tr>
<tr>
<td>Polynucleotide phosphorylase (PNPase)</td>
<td>pnp</td>
<td>-7</td>
<td>Exonuclease (3' → 5')</td>
<td>mRNA, RNAI</td>
</tr>
<tr>
<td>Poly(A) polymerase (PAP I)</td>
<td>pcnB</td>
<td>Deletion</td>
<td>Adenylation</td>
<td>mRNA, RNAI</td>
</tr>
<tr>
<td>Ribonuclease II (RNase II)</td>
<td>rnb</td>
<td>Deletion</td>
<td>Endonuclease (double strand)</td>
<td>rRNA, mRNA, RNAI</td>
</tr>
</tbody>
</table>

1986; March et al., 1989; Liu & Parkinson, 1989; also reviewed by Sarkar, 1996, and Cohen, 1995, has since been implicated in mRNA decay (O'Hara et al., 1995; Hajnsdorf et al., 1993). Its central role in RNAI decay was demonstrated by both Cohen's group and our own (Xu et al., 1993; He et al., 1993).

Here we investigate whether the two remaining enzymes believed to have major roles in mRNA processing are implicated in RNAI decay. We present the first evidence that RNase III, an endonuclease involved in both the processing of rRNA (Dunn & Studier, 1973) and the degradation or processing of a variety of messages (see Kushner, 1996, for references), also can affect RNAI degradation. On the other hand, RNase II, the second known enzyme involved in mRNA decay based on the half-lives of the primary and processed forms of RNAI in strains lacking one or more decay enzymes.

METHODS

Bacterial strains and plasmids. All experiments described were carried out using congenic strains constructed from Escherichia coli MM38 (Masters et al., 1993). Mutations in rne, rnc, pnp, rnb and pcnB were transferred into MM38, mainly by P1 transduction. The alleles used here and their sources are as follows: pcnBΔ-26 is from MM38K26 (Masters et al., 1993); pnp-7 is from SK7262 (Yancey & Kushner, 1990) and was originally isolated by Reiner (1969); rnbΔ-201 (Tet4) is from CMA201 (Piedade et al., 1995); rnb-500 (Ts), used in strain verification, is from SK6632 (Yancey & Kushner, 1990) and was first described by Donovan & Kushner (1986); rne-1 (Ts) is from HAK117 (Ono & Kuwano, 1979); rne-311 (Ts) is from N3431 (Apirion, 1978); and rne-3A-8 (Kan8) is from SK7621 (Babitzke et al., 1993). To move interrupted genes [rneΔ-38 (Kan8); rnbΔ-201 (Tet4); pcnBΔ-26 (Kan8)] the antibiotic-resistance marker was used for selection. pnp-7 inheritance was screened after selection of the linked argG+ marker. A linked Tn10 (Tet4) was transferred into the rne (Ts) rnb (Ts) and pcnBΔ strains to facilitate the transfer of these alleles. In order to construct MM38 rne-3071 rneΔ pcnBΔ it was necessary to introduce the pcnB allele by conjugation. In cases where the inheritance of particular alleles in certain combinations could not be ascertained by phenotypic tests (i.e. resistance to a drug could be conferred in more than one way), backcrosses (P1 transduction) were used to verify constructions.

The following plasmids were used: pJF118EH (Furste et al., 1986), pBR325 (Bolivar, 1978), pCML108 (Lin-Chao et al., 1994), pLH1c (He et al., 1993) and pBAD-PCN. pBR325 and pJF118EH, closely similar pBR322-based replicons, were used interchangeably to produce RNAI. pCML108 is a pSC101-based replicon from which RNAI, but no other ColE1-derived product, is expressed. In pLH1c, pcnB is cloned downstream of ptac (which is leaky); however, it is also constitutively expressed from an upstream chromosomal promoter which is weaker than the native pcnB promoter; the native promoter is not present in this construct (N. Binns, unpublished). pBAD-PCN is a plasmid in which pcnB expression is under the control of the arabinose-inducible pBAD promoter. AraR (0.2 or 0.4%) was added to induce the production or increase the concentration of PAP I during the course of an experiment. Expression from this promoter is almost completely inhibited by growth in the presence of 0.2% glucose. pBAD-PCN was constructed by cloning 1460 bp PCR-amplified pcnB DNA (from pJMS1; Masters et al., 1989) into the EcoRI and HindIII sites of pBAD18 (Guzman et al., 1995). The primers used were (upstream) 5'-GCTAT GATTA GCCGG AATTC TTTTG and (downstream) 5'-GTGCCT TATGG TCCAC TTCGTG-3' and were then transferred to positively charged nylon membranes.

RNAI half-life measurements. Cells were grown at 37 °C in L-broth, treated with rifampicin (RIF; 0.25 mg ml−1; Sigma) to stop further RNA synthesis and sampled at intervals. RNA was extracted, separated by size on polyacrylamide gels, transferred to a nylon membrane and Northern blots were made by hybridizing radioactively labelled oligonucleotide probes, specific for RNAI or for a tRNA control, to the membrane, all as described by He et al. (1993), except that RIF was added when the OD600 reached 0.4. Strains with temperature-sensitive mutations (rne-1 and rne-3071) were grown at 30 °C to an OD600 of 0.3 and were then transferred to 44 °C and grown for a further 60 min prior to the addition of RIF. tRNA is assumed to be stable and acts as a loading standard. The time-dependent change in RNAI/tRNA was used to calculate RNAI half-life graphically.

Preparation of RNA and Northern blot analysis. The protocols used for the extraction of RNA and Northern blot analysis were as described by He et al. (1993) with some changes. RNA was separated by size on polyacrylamide/7 M urea denaturing gels and transferred to positively charged nylon membranes.
(Boehringer Mannheim) and fixed to the membrane by UV cross-linking, using a Stratagene UV Stratalinker at 1200 µJ, 254 nm. Oligonucleotide probes were end-labelled with 32P using New England Biolabs polynucleotide kinase and following the manufacturer’s protocol; hybridization was carried out according to Church & Gilbert (1984). Hybridization signals were quantified using a Molecular Dynamics Phosphor Imager 400S (Molecular Dynamics) and Image-Quant version 3.22 software. Autoradiographs were made using Kodak X-Omat film. Hybridizations using the UB6 oligonucleotide to detect 3’ polyadenylation were done at 40 °C; all others were done at 50 °C. Filters requiring a second hybridization were stripped overnight in 100 ml 50 mM Tris/HCl (pH 8/0), 0.1 mM EDTA, 0.1 % SDS at 65 °C.

Oligodeoxyribonucleotides. Oligonucleotides were purchased from Oswel DNA Service. The probe used to detect RNAI and for primer extension analysis was UB2 (5’-GATCA AGAGC TACCA ACTCT T-3’). The RNA2 control probe, SS2 (He et al., 1993), was 5’-CCGGT AGAGT TGCCC CTACT CCGGT TTGTG-3’. Primers for the RT-PCR reaction were UB2 and UB1 (5’-ACAGT ATTTG GTATC TGCGC TCTGC-3’) for the control reaction and UB6 (5’-TTTTT TTGTT TTTTTTTA AAAAAA CACC-3’) and UB1 to establish the presence of adenylation.

Primer extension analysis. Primer extension analysis/reverse transcription was performed on total RNA extracted from cells harbouring the pBR325 plasmid. The method used was from Current Protocols in Molecular Biology (Triezenberg, 1992), and used 2–4 units AMV reverse transcriptase (Promega AMV; 5–10 units µl-1) and 10 pmol 32P-labelled primer (T4 kinase; New England Biolabs), labelled as described by the manufacturer. Unincorporated nucleotide was removed using NAP-5 columns (Pharmacia). One-third of the reaction mix was loaded on an 8 % polyacrylamide/7 M urea gel. Primer extension products were sized using a DNA sequencing ladder generated with a Pharmacia T7 Sequencing kit and the supplied M13 template.

RT-PCR. cDNA was generated from total RNA extracts using the UB6 or UB2 primer and AMV reverse transcriptase at 40 °C using the same methods as described for primer extension except that the primers were not labelled. Twenty-six picomoles of additional primer (UB2 and UB1 for the control reaction or UB6 and UB1 to amplify polyadenylated sequences) was added to 10 µl of the cDNA reaction product along with 200 µmol dNTPs, 1.5 mM MgCl2 and 2–5 units Taq polymerase (Promega Taq 5 units µl-1) in a final volume of 100 µl Taq buffer. A Hybaid Omnigene thermocycler and the following cycling conditions were used: 1 cycle at 94 °C for 3 min, 50 °C for 30 s and elongation at 72 °C for 1 min followed by 30 cycles of 94 °C for 1 min, 50 °C for 30 s and elongation at 72 °C for 1 min. This was followed by a final elongation step at 72 °C for 10 min. The PCR products were separated on a 1.25 % agarose gel, in TBE buffer (0.089 M Tris/borate, 0.002 M EDTA). The amount of ethidium-stained product was measured directly using a Transilluminator and a UVF camera gel analysis system or a Southern blot was analysed using the Phosphor-Imager. The ratio of PCR products generated by the two primer pairs from a single RNA sample was taken as a measure of relative polyadenylation.

In vitro RNase III cleavage. In vitro cleavage of total RNA samples prepared as described above was performed using His-tagged RNase III and the method described by Li et al. (1993), with or without potassium glutamate. The purified RNase III, the generous gift of A. Nicholson (Wayne State University, Detroit, MI, USA), had a concentration of 0.14 µg µl-1. The final reaction volume of 20 µl contained 5–10 µg RNA and 3 µl RNase III. The reaction was incubated at 37 °C for 0, 10, 20 and 30 min and stopped with stop buffer (50 % deionized formamide, 20 mM EDTA, 89 mM Tris/HCl, pH 7.5, 89 mM boric acid, 20% sucrose and 0.1 % each of bromophenol blue and xylene cyanol). Half of each reaction mix was loaded onto an 8 % polyacrylamide/7 M urea gel and transferred to nylon as described above. Hybridization with an RNAI-specific probe was used to reveal the RNase III cleavage pattern of RNAI synthesized from pBR325 or pCML108.

RESULTS

RNAI decay involves RNase E, PAP I and PNPase, but not RNase II

RNAI is 108 nt long and adopts a structure with three stem–loops and an unpaired 5’ region of 9 bases (see Fig. 1); it is unstable in vivo (Elble et al., 1983) and we find, in confirmation of earlier reports (Lin-Chao & Bremer, 1986; Brenner & Tomizawa, 1991; Lin-Chao & Cohen, 1991; He et al., 1993), that it decays rapidly with a half-life of about 2 min at 37 °C (Table 2).

The absence of RNase E, PAP I and PNPase has previously, as cited above, been shown to slow RNAI decay and we confirm this here (Table 2 lists the half-lives of RNAI in RNA-decay mutants). Fig. 2(a, b and d) are Northern blots which show the decay of RNAI in particular mutant strains. In the absence of PAP I (and RNAI) RNAI103, the primary product of RNase E processing, and some shorter processed forms of lengths ~ 93 (Fig. 2a, lane 1; this band is obscured by rRNA in lanes 2–7), ~ 83, ~ 73/71 and ~ 65 can be observed. RNAI103, the principal processed RNAI product and barely detectable in pcnB+ hosts, has a half-life of 25 min, as previously observed. The 65 nt band appears as the 83 nt band disappears (lanes 4–7), suggesting that it may originate from it. The stable doublet labelled 73/71 is probably identical to the 70/67 pair identified as minor products of RNase E action (Kaberlin et al., 1996). We calculate that, at the time of RIP addition, these shorter forms, together, have about one-third the abundance of RNAI103. The RNAI analysed here was transcribed from pCML108, which does not have the RNAII coding sequence. We find that, in the absence of RNAII and PcnB, a condition not previously analysed, these decay bands are particularly easy to observe, suggesting that RNAI:RNAII hybrids are probably degraded more efficiently than is folded RNAI.

In a PNPase-deficient strain a diffuse band of material, varying upwards in length from 103 nt, is apparent (Fig. 2b, lane 6). Reverse transcription analysis shows that most of this material has the RNase E processed 5’ end (Fig. 2c; and Xu & Cohen, 1995) and thus must be lengthened at the 3’ end. Its absence in a pcnB pap7 strain (Fig. 2b, lanes 1–5) suggests that it represents adenylation material. These confirmatory results are included here for comparative purposes.
To test whether RNase II has any significant role in RNAI decay we measured the half-lives of RNAI_{108} in MM38 (Fig. 2d) and RNAI_{103} in MM38 pcnB rnb (Northern blot not shown). RNAI_{108} decays at about the same rate in the presence or absence of RNase II (Fig. 2d; and Table 2, rows 1 and 5); the rate of RNAI_{103} decay is also unchanged in the absence of RNase II (Table 2, rows 3 and 7).
A second, RNase-E-independent, path of RNAI decay involves RNase III

Fig. 3 shows Northern blot analyses of RNAI decay in rne and rnc strains. Fig. 3(b) (top curve) shows that RNAI$_{108}$ decays in MM38 rne-3071 with a half-life of ~8 min when the enzyme is inactivated at 44°C, fourfold slower than in non-mutant cells (see also Lin-Chao & Cohen, 1991). Although some RNAI$_{108}$ remains even 60 min after RIF addition (Fig. 3a), it is clear that RNAI can be degraded independently of RNase E. To test whether this might require RNase III, the second principal endonuclease with a known role in mRNA processing, we constructed an rnc rne-3071 double mutant. Fig. 3(c) shows that, in MM38 rnc rne-3071, significant amounts of RNAI remain at 60 min after RIF addition, but principally as a novel form of increased length (RNAI$_{ex}$), rather than as RNAI$_{108}$. The half-life of total RNAI is increased to 33 min in the double mutant. In an rne$^+$ rnc strain RNAI$_{ex}$ also accumulates (Fig. 3d), suggesting that it is not a substrate for RNase E. We conclude that RNase III is required either to remove, or to prevent the formation of, RNAI$_{ex}$.

Reverse transcription analysis of RNA extracted from an rnc strain after 60 min with RIF showed that the remaining RNAI (virtually all RNAI$_{ex}$; Fig. 3d) has a native 5’ end (Fig. 2c, rt.). Therefore RNAI$_{ex}$ is made by extending RNAI$_{108}$ at the 3’ end of the molecule. Fig. 3(d) also shows that RNAI$_{103}$ (absent from rne strains) decays in rnc strains at a moderately reduced but still rapid rate (Table 2; Fig. 3b, lowest curve). Therefore RNase III is not important for the adenylation-dependent decay of this RNase E processed form. Note that the mean length of RNAI$_{ex}$ increases after RIF treatment, reaching a maximum of ~116 nt at about 16 min. After longer times the mean length again decreases.

Closer examination of the kinetics of RNAI decay in rnc strains shows that the fates of RNAI$_{108}$ and RNAI$_{ex}$ are very different. This is shown in Fig. 3(b) in which the decay of RNAI$_{108}$ + RNAI$_{ex}$ (middle curve) is plotted.
The complex shape of this curve results from the initial (most likely RNase E mediated) rapid decay of RNAI* combined with the conversion of a substantial fraction of RNAI* into the much more stable RNAI ex. RNAI ex has an approximate half-life of 45 min in this experiment.

**RNAI ex is likely to be PAP I adenylated**

The longer form of RNAI, RNAI ex, which appears in rnc strains is likely to be adenylated RNAI*. Observations which suggest this are shown in the Northern blots in Fig. 4(a–c). Firstly, extended RNAI* is not observed in an rnc pcnB strain (Fig. 4a), indicating that PAP I activity is required for it to be made. Conversely, if PAP I is overexpressed in an rnc strain an increasing proportion of extended RNAI is observed as PAP I concentration increases (Fig. 4b). In the experiment shown, the production of PAP I was induced by addition of arabinose to MM38 rnc (pBAD-PCN), a strain in which PAP I production is under the control of the araBAD promoter. Overproduction of PAP I also promotes the production of extended RNAI in rnc+ strains; this material is not long-lived (Fig. 4c).

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**Fig. 3.** Decay of RNAI in RNA endonuclease mutants. (a, c, d) Northern blots of RNA extracts of the indicated mutants sampled at the noted times relative to RIF addition. Methods and probes were as for Fig. 1; all strains contained pBR325. RNAI markers (labelled M), where included, were prepared by mixing extracts of plasmid-containing pcnB and pcnB+ strains to ensure that both RNAI* (the predominant form in pcnB strains) and RNAI* (the predominant form in pcnB+ strains) would be visible. rne strains were grown at 30 °C and transferred to 44 °C 1 h before RIF addition. (a) MM38 rne3071; 0 and 60 min samples are shown in this panel, but values for all points up to 30 min are plotted in (b). (b) Decay curves for RNAI from rne and rnc strains. The upper line (rne) shows RNAI* decay in MM38 rne-3071 ( ), the middle line (rnc) shows the decay of RNAI* ( ). Calculated RNAI half-lives (t1/2) are shown. (c) RNAI decay in MM38 rne-3071 Δrnc. (d) RNAI decay in MM38 Δrnc grown at 37 °C.
To demonstrate that RNAI\textsubscript{ex} is adenylated, RT-PCR amplification was carried out using UB6, a primer which includes nine 5' dT residues, for selective reverse transcription of adenylated RNAI. The primer UB2, which is not dT-rich, was used in a parallel reaction as a control. Fig. 4(d) shows an EtBr-stained agarose gel of the PCR-amplified products obtained in this way. Significant amounts of UB6 primed amplified product were obtained only with RNA from control strains carrying the \textit{pnp-7} mutation, or overexpressing PAP I from pLH1c, in which adenylation is known to be enhanced, thus validating the technique. The amount of RT-PCR product obtained in this way from the \textit{rnc} strain was as great as that produced from the hyperadenylating control strains.

Direct Northern blotting of RNAs from different mutant strains was also done using \textsuperscript{32}P-labelled UB6 and UB2 oligonucleotides as probes. Fig. 4(e) shows that the UB6 probe, designed to detect adenylated RNAI, hybridized preferentially to the RNAI extracted from the \textit{rnc} mutant or from the PAP I overproducer. The control probe hybridized to all forms of RNAI in each strain. This is the result expected if UB6 is specific for polyadenylated RNAI and if, amongst the strains tested here, RNAI is only extensively polyadenylated in the RNase-III-deficient and PAP-I-overproducing strains. Taking all of the above evidence together, we conclude that RNAI\textsubscript{ex} is likely to be a 3' adenylated form of RNAI\textsubscript{108} (to be referred to as RNAI\textsubscript{108A}) which is produced by PAP I adenylation of RNAI\textsubscript{108.}
Is RNAI a substrate for RNase III cutting?

The experiments described above demonstrate that a stable, adenylated form of RNAI_{108} accumulates in the absence of RNase III. This could mean that either RNase III itself cuts RNAI_{108}, or RNase III works indirectly by, for instance, facilitating the production of the actual degrading enzyme. To distinguish between these possibilities we attempted to identify possible products of RNase III cleavage in vivo and in vitro. Our results, although not conclusive, show that RNase III can cut RNAI, but cannot define an in vivo substrate.

Possible products of RNase III cutting in vivo

Since the degradation products of adenylated RNAI disappear too rapidly to be observed on Northern blots we examined the more stable processed forms of unadenylated RNAI extracted from pcnB strains to see whether their presence is RNase III dependent. Fig. 5(a) shows that two bands of \( \approx 93 \) nt and \( \approx 83 \) nt in size, visible in pcnB extracts, are absent from pcnB rnc extracts, and are thus candidate products of RNase III cutting. We also examined pcnB rne extracts since they lack both major enzymes thought to initiate RNAI decay. The Northern blot in Fig. 5(b) shows extracts of MM38 rne-1 pcnB sampled after RIF inhibition of RNA synthesis. As RNAI decays, a \( \approx 95 \) nt degradation product of RNAI appears. The band is broad and could contain a mixture of the products of RNAI_{108} and RNAI_{108} cut at or near nt 98. The kinetics of disappearance of RNAI_{108} and RNAI_{108} and of the appearance of the \( \approx 95 \) nt form (data not shown) is consistent with this interpretation.

Curiously, the combination of rne-1 and rnc was not tolerated in either MM38 or in MG1655, preventing us from testing whether the formation of the 95 nt form is dependent on RNase III in an rne-1 mutant. However, MM38 rne-3071 rnc pcnB and MM38 rne-3071 pcnB could be constructed and RNAI decay was followed in this congenic pair of strains. No diffuse band at \( \approx 95 \) nt is seen when RNase III is absent (Fig. 5c, upper) but RNAI of this length is observed when RNase III is available (Fig. 5c, lower).
RNase III can cut RNAI in vitro

If RNase III were able to cut RNAI in vitro, in the absence of any other enzymes of RNA metabolism, to yield products of the same size(s) as the RNase-III-dependent bands identified in vivo this would support the hypothesis that RNase III can cut RNAI in the cell. Protein-free extracts of RNA were prepared from MM38 pcnB (pBAD-PCN) grown with either arabinose (to induce PAP I production) or glucose (to repress production) and digested with purified RNase III using a standard protocol (Li et al., 1993). No cutting of RNAI was observed (data not shown). However, when the salt (potassium glutamate), generally considered to be required for specificity of cutting, was omitted from the reaction mixture, cutting was observed. Fig. 6(a) shows a Northern blot of samples taken during RNase III treatment. All forms of RNAI present are fully and specifically processed to yield two major products each. Since the 103 nt and 108 nt forms are cut to yield products differing in size by 5 bases, the sites of cutting must be near the 3' rather than near the 5' end of RNAI, with the longer product resulting from a cut near nt 98 and the shorter from a cut near nt 82 (indicated in Fig. 1). When RNAI is folded these bases are close together in a double-stranded region flanking two GC pairs which are in turn flanked by AU pairs. This putative cutting site resembles that identified in rncO (Matsunaga et al., 1996; Bardwell et al., 1989). The material in the 73/71 doublet band prominent in the absence of PAP I is also rapidly processed by RNase III.

To see whether either of the RNase III products observed in vitro correspond in size to RNase-III-dependent in vivo products, the extract used in Fig. 5(a) was run alongside of the in vitro cut material. This is shown in Fig. 6(b). A product 93 nt in length is found in both extracts and thus could be an in vivo product of RNase III digestion.

Fig. 6. (a) In vitro cutting of RNAI extracted from MM38 ∆pcnB (pBAD-PCN) grown with 0.4% arabinose (indicated on top line) or 0.2% glucose. Purified, deproteinized RNA was cut with RNase III at 37 °C under low-salt conditions, as described in Methods, for the number of minutes indicated. RNAI and its fragments were detected by hybridization with UB2 and sized with reference to a sequencing ladder. (b) Northern blots to compare sizes of RNAI decay products extracted from cells with those produced by RNase III cleavage in vitro. Run on a single gel are: extract of MM38 ∆pcnB (pCML108) (lane 1); MM38 ∆pcnB (pBAD-PCN) RNase III cleaved samples (lanes 2–5) (also shown on the gel in Fig. 6a) grown + ara (lanes 2–3) or – ara (+ glu) (lanes 4–5). Length of incubation (min) with RNase III is shown at the bottom.

RNase-III-dependent cutting of RNAI does not require RNAII

RNase III cuts both double-stranded RNA and folded single-stranded RNA. Since RNAI forms a hybrid with RNAII it is possible that this hybrid, rather than RNAI, is the primary substrate for RNase III. Indeed, Tomizawa & Itoh (1981) reported that this was the case in vitro. As there is less than 10% as much RNAII as RNAI in broth-grown cells, most RNAI will be unhybridized, making it unlikely that only RNAI:RNAII hybrid is being cut. However, to confirm that RNAII is not required for RNase III cutting of RNAI, we repeated several of the experiments described above using pCML108 (Lin-Chao et al., 1994), a pSC101 derivative which produces RNAI but not RNAII, to supply RNAI. RNAI extracted from pCML108 strains can also be cut by RNase III in vitro to yield fragments of the sizes described above. Extended RNAI also accumulates in an rnc strain with this plasmid (data not shown), showing that hybridization with RNAII is not required to produce material which requires RNase III for its removal.

DISCUSSION

Is the in vivo role of RNase III in RNAI breakdown direct or indirect?

We have shown that, in the absence of RNase III, an extended, adenylated, stable form of RNAI, RNAI_{adenylated}, is made. We can think of three possible origins for RNAI_{adenylated}: (1) Lack of RNase III could itself stimulate the formation of RNAI_{adenylated}, a form not ordinarily made at all, and for which there is no mechanism of decay. We do not think that this is so because when adenylation of RNAI is artificially increased by over-expressing pcnB in rnc strains, RNAI_{adenylated} is formed but does not have an increased half-life (Fig. 4c). (2)
Lack of RNase III promotes the formation of an inhibitor that interferes with the degradation of RNAI\(_{108}A_n\), or prevents the formation of an unidentified nuclease that normally would degrade it. We cannot exclude these possibilities (which would themselves be interesting), but it is worth noting that the level of a candidate nuclease, PNPase, is increased rather than decreased in rnc mutants (Portier et al., 1987). (3) RNase III has a direct role in RNAI degradation, that of initiating the decay of RNAI\(_{108}A_n\).

Distinguishing between (2) and (3) depends on determining whether RNAI (or RNAI\(_{108}A_n\)) is a bona fide in vivo substrate of RNase III. We have not been able to do this here. What we have shown is that lack of RNase III leads to the accumulation of a novel form of RNAI (a possible substrate?) and that certain RNAI decay products are not observed when RNase III is absent (possible products?).

An in vitro demonstration that RNase III could cut RNAI to produce the products observed in vivo would support the hypothesis that RNAI is an RNase III substrate in the cell. Our in vitro results show very specific cutting by RNase III, but only at what are normally regarded as ‘low-specificity’ sites (Li et al., 1993; Dunn, 1976), that is, sites cut only when ionic strength is low. However, since RNase III cleavage of RNAI is not a preferred reaction in vivo, and indeed appears to occur principally on adenylated substrates or in the absence of RNase E or PAP I, inefficient cutting could ensure that RNase III processing does not take precedence over that carried out by RNase E. It is interesting to note that RNase III, which cuts p10Sa RNA in vivo, only cuts this substrate in vitro in low salt in a reaction requiring Mn\(^{2+}\) but inhibited by Mg\(^{2+}\) (Srivastava et al., 1992). This shows that special conditions can be required for RNase III cutting of particular substrates and that the existence of a cellular factor that promotes RNase III cutting of adenylated RNAI cannot be excluded. Taken together our results are consistent with the possibility, but do not prove, that RNAI is a substrate for RNase III.

A loose consensus sequence has been suggested for RNase III recognition (Krinke & Wulff, 1990) but it remains difficult to identify a potential substrate on the basis of sequence alone. RNase III can cut the stems of folded single-stranded RNA molecules; although RNAI has three stem–loops these are rather shorter than the stems which have principally been described as RNase III substrates. However, Matsuura et al. (1996) have reported that a much reduced stem in rncO, shorter than the RNAI stems, remains a substrate, although a less efficient one, for RNase III cutting.

A suggested pathway for RNAI decay which includes RNase III

Based on the observations reported here and elsewhere we propose that these are the major reactions leading to RNAI decay (Fig. 7).

(1) The initial processing of RNAI\(_{108}\) is carried out either by RNase E or PAP I.

(2) RNAI\(_{108}A_n\) is not converted to RNAI\(_{108}A_n\) by RNase E, but is instead broken down by reactions dependent on RNase III. Fig. 1 shows a possible structure for RNAI\(_{108}A_n\), based on the assumption that some of the 3’As can pair with 5’Us. (It is interesting to note that the longest forms of RNAI we observe, ~117 nt, are equivalent to 1 A per unpaired 5’ nucleotide.) RNAI\(_{108}A_n\) is not readily removed by exonucleases; in the absence of RNase III it remains stable. This is particularly surprising in view of the fact that PNPase is increased 10-fold in rnc strains.

(3) RNAI\(_{103}\) is converted to RNAI\(_{103}A_n\) by PAP I. That this reaction can be carried out independently of RNase E cutting was shown by inducing PAP I in a ΔpcnB strain. Accumulated RNAI\(_{103}\) then disappeared rapidly (data not shown).

(4) PNPase promotes RNAI\(_{103}A_n\) decay. In the absence of PNPase, RNAI\(_{103}A_n\) accumulates (Xu & Cohen, 1995; and Fig. 1). That PNPase can work independently of RNase E is shown by the fact that pre-existing RNAI\(_{103}\) is rapidly degraded after PAP I induction.

Thus we suggest that RNAI decay can proceed by one of two principal routes, both of which require that it be adenylated by PAP I. The primary pathway of decay starts with RNase E cleavage, and is followed by PAP-I-mediated adenylation and PNPase-mediated exonucleo-
lytic decay. The secondary route begins with adenylation of RNAI$_{5p}$. Since RNase E does not appear to cleave adenylated RNAI$_{108}$, RNase III is required. In a $pcnB rne$ mutant, from which RNase E, RNase III and PAP I are absent, RNAI$_{108}$ none the less continues to disappear. There thus must be other enzymes which are able to initiate its decay.

**Implications for mRNA decay**

Several of the observations we have made here may have implications for the way in which RNAs other than RNAI turn over. Firstly, each step in RNAI decay seems to require a specific enzyme which does not appear to be replaceable by another enzyme with similar activity. Thus we see that although either RNase II or PNPase is required for cell viability (Donovan & Kushner, 1986), suggesting that they can substitute for one another, they do not appear to be able to do so here. Presumably the strong 3' stem-loop prevents RNase II action, possibly by denying it an anchor (Cannistraro & Kennell, 1994), while added A residues offer a 'handle' for PNPase attack (Xu & Cohen, 1995). Secondly, 3' adenylation is not necessarily sufficient to allow PNPase attack; adenylated RNAI$_{108}$ appears very resistant. This could in part be because the 5'ppp interferes with PNPase activity, as has been reported for 5pppRNAI$_{103}$ (Xu & Cohen, 1995) and RNase E unprocessable RNAI$_{108}$ derivatives (Bouvet & Belasco, 1992), but the degree of resistance suggests another cause. One possibility is that, although this would not be a strong interaction, the 5' A-tract base-pairs with the 3' single-stranded region to sequester both the RNase E cutting site and the single-stranded 3' end (Fig. 1). Although a mRNA, because it is much longer than RNAI, is likely to be susceptible to several alternative decay routes (Haugel-Nielsen et al., 1996; Coburn & Mackie, 1996), any given subsegment may be as restricted as is RNAI in the way in which it can be degraded. Several associations of enzymes which may expedite RNA decay have been described. The 'degradosome' is a copurifying group of enzymes, which include RNase E and PNPase, with RNA-degrading activity. It has been suggested that the absence of RNase E or PNPase may strongly reduce the rate at which a cell can act on its RNAI substrate (Xu & Cohen, 1995). We do not find evidence of that here. Although the half-life of RNAI is increased in $pnp$ mutants, the stable material has been processed by RNase E; there is no accumulation of RNAI$_{108}$ unprocessed by RNase E, in $pnp$ $pcnB$ mutants. Furthermore, digestion of RNAI$_{108}$ by PNPase can be separated in time from RNaseE action (by inducing PcnB production in a $pcnB$ background). Thus although endo- and exonucleases may well be associated in the cell, effective in vivo activity does not require coordinate action.

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**REFERENCES**


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