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A ribonuclease specific for inosine-containing RNA: a potential role in antiviral defence?

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RNA transcripts in which all guanosine residues are replaced by inosine are degraded at a highly accelerated rate when incubated in extracts from HeLa cells, sheep uterus or pig brain. We report here the partial purification and characterization of a novel ribonuclease, referred to as I-RNase, that is responsible for the degradation of inosine-containing RNA (I-RNA). I-RNase is Mg^{2+} dependent and specifically degrades single-stranded I-RNA. Comparison of the K_m of the enzyme for I-RNA with the K_i for inhibition by normal RNA suggests a ~300-fold preferential binding to I-RNA, which can account for the specificity of degradation. The site of cleavage by I-RNase is non-specific; I-RNase acts as a 3′→5′ exonuclease generating 5′-NMPs as products. The presence of alternative unconventional nucleotides in RNA does not result in degradation unless inosine residues are also present. We show that I-RNase is able to degrade RNAs that previously have been modified by the RED-1 double-stranded RNA adenosine deaminase (dsRAD). dsRADs destabilize dsRNA by converting adenosine to inosine, and some of these enzymes are interferon inducible. We therefore speculate that I-RNase in concert with dsRAD may form part of a novel cellular antiviral defence mechanism that acts to degrade dsRNA.

Keywords: inosine/ribonuclease/RNA modification/RNA stability/RNA turnover

Introduction

The processing and turnover of various classes of RNA in prokaryotic and eukaryotic cells involves multiple steps in which ribonucleases (RNases) play a role. Consequently, it is not surprising that there are a large number of distinct RNases to fulfil these roles (reviewed in Deutscher, 1993; Beelman and Parker, 1995). Among the criteria by which RNases can be distinguished are their action as endo- or exonucleases, whether they cleave only adjacent to specific bases, whether they are specific for a particular sub-class of RNA such as rRNA, whether they act upon double- or single-stranded RNA and whether they cleave phosphodiester bonds to leave 5′ or 3′ nucleotide monophosphates (NMPs). In addition, some RNases, such as RNase L (see below), are not constitutively active, but exhibit inducible activity. As well as the more well known roles of RNases in turnover of mRNAs and processing of tRNA and rRNAs, they are also used in various circumstances by bacterial, plant and mammalian cells as cytotoxic agents to kill cells selectively. Some such cytotoxic nucleases have even been tested for use in anti-cancer therapy (Wu *et al.* 1993).

In the course of investigating a proposed non-Watson–Crick base pairing interaction between the terminal guanosine residues in pre-mRNA introns, we tested the splicing activity of RNA transcripts in which all of the internal guanosine residues had been replaced by inosine (Scadden and Smith, 1995). Inosine is identical to guanosine except for the lack of the exocyclic amine group at position 2 of the purine ring. Unexpectedly, when incubated in HeLa cell nuclear extracts, the inosine-containing RNAs (I-RNAs) appeared to be considerably less stable than the guanosine-containing transcripts (G-RNAs). This observation was repeated subsequently with several other unrelated RNAs, many of which were not splicing substrates. These observations indicated that the instability of the I-RNAs was not attributable to increased accessibility to degradation by general RNases due to failure of the I-RNA to be incorporated into spliceosomes. In addition, the various substrates were not predicted to contain strong secondary structure which may have been weakened by inosine substitution leading to preferential degadation. These data therefore suggested that HeLa nuclear extracts contain a nuclease activity that is specific for I-RNA.

Inosine occurs naturally in several places within cellular RNA. One well-characterized site is in the ‘wobble’ position of some tRNA anticodons (Elliot and Trewyn, 1984). Inosine has also been detected *in vitro* as the result of hydrolytic deamination of adenosine residues in double-stranded RNA (dsRNA) by dsRNA adenosine deaminases (dsRAD; Bass and Weintraub, 1988; Wagner *et al.*, 1989). The conversion of A–U to I–U base pairs by dsRAD results in destabilization of the substrate dsRNA. Such A→I changes appear to have a role in at least some cases of site-specific RNA editing. For example, the editing in the brain of mRNA encoding the glutamate receptor subunit B (GluRB) involves the formation of double-stranded structures around the editing site (Higuchi *et al.*, 1993; Maas *et al.*, 1996) and can be catalysed *in vitro* by either purified dsRAD (Hurst *et al.*, 1995; Dabiri *et al.*, 1996; Maas *et al.*, 1996) or a related enzyme, RED-1 (Melcher *et al.*, 1996). Inosine is recognized as G by the translation machinery so what was originally a glutamine codon is recognized as arginine after editing. Pure *Xenopus* dsRAD has also been shown to edit hepatitis delta virus RNA efficiently *in vitro* (Polson *et al.*, 1996).

Despite the accumulation of evidence for a role in RNA editing, expression of dsRAD activity is much more widespread than the pre-mRNAs that it is known to edit (Wagner *et al.*, 1990; Higuchi *et al.*, 1993), suggesting the likelihood of a more general role for the enzyme. A clue to a less specialized function is that levels of dsRAD

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increase in response to interferon (Patterson and Samuel, 1995; Patterson *et al.*, 1995). Interferon induction and activation by dsRNA are hallmarks of enzymes that are involved principally in cellular antiviral defence (Samuel, 1991). The significance of activation by dsRNA is that viral infection often involves the production of high levels of dsRNA as a consequence either of viral replication or of transcription from opposite strands of DNA viruses. For instance, protein kinase R (PKR) is induced by interferon and activated by dsRNA in an autophosphorylation reaction (Clemens, 1996). Activated PKR phosphorylates the translation initiation factor eIF2α resulting in a shut-down of protein synthesis in virally infected cells. Likewise, oligo 2′-5′ A synthetase is interferon inducible and activated by dsRNA (Witt *et al.*, 1993). Its product, 2′-5′ oligo A, is an activator of RNase L, which degrades single-stranded RNA (ssRNA) in virally infected cells. Therefore, dsRAD, as an enzyme that is interferon inducible and that acts upon dsRNA, probably plays a role in cellular antiviral defence. Multiple A→I conversions would cause miscoding of viral messages. Nevertheless, the viral RNAs potentially would still clog up the cellular translation machinery. An RNase specific for I-RNA potentially could play an important role in disposing of the products of dsRAD, producing a more effective and streamlined antiviral defence pathway. Based upon this speculation, we have sought to characterize the properties of the I-RNase activity that we detected initially in HeLa nuclear extracts (Scadden and Smith, 1995).

Here we report the partial purification and characterization of I-RNase activity from pig brain. We show that I-RNase is an Mg^{2+}-dependent 3′→5′ exoribonuclease which has specificity for single-stranded I-RNA. The presence of alternative unconventional nucleotides in RNA does not result in degradation unless inosine residues are also present. The specificity of I-RNase is not determined by the site of cleavage since the products of I-RNase cleavage are predominantly 5′-NMPs. Rather, the specificity appears to arise at the level of binding to RNA with an ~300-fold higher affinity for I-RNA than for G-RNA. Finally, we show that I-RNase is able to specifically degrade RNA that has been modified by the enzyme RED-1. I-RNase, therefore, appears to be a novel RNase whose properties are consistent with a role in cellular viral defence.

Results

Our original observation of enhanced degradation of I-RNA in HeLa nuclear extracts (Scadden and Smith, 1995) has been repeated consistently with a wide variety of transcripts (see Figures 1–6 for use of various RNAs). We subsequently have detected I-RNase activity in crude extracts made from sheep uterine smooth muscle and pig brain (Figure 1A), which suggests that I-RNase is a widespread activity at least in mammalian tissues. All the subsequent experiments described here used I-RNase which has been partially purified from a pig brain extract. Partial purification of I-RNase involved three chromatographic steps, and has yielded a fraction which has high I-RNase activity and is free of other general nucleases (see Materials and methods).

The basic assay for I-RNase activity is illustrated in Figure 1B. The transcript ΔKP contains exons 2 and 3 of the rat α-tropomyosin gene with no intron between (Smith and Nadal-Ginard, 1989). The ΔKP template was linearized with either *Bam*HI or *Bgl*II, which enabled synthesis of transcripts of two different lengths which contained either guanosine or inosine (G-RNA or I-RNA). The G-RNA transcript was ~50 nucleotides longer than the I-RNA transcript and served as an internal control for assessing degradation of I-RNAs in the same tube. Figure 1B shows a time course of degradation of I-RNA and G-RNA ΔKP transcripts. Quantitation by phosphorimaging indicated that I-RNA transcripts were degraded at a rate of 469 ± 23 ng RNA/min/mg protein (n = 3), while the G-RNA transcripts were degraded at a rate of 18.8 ± 0.6 ng RNA/min/mg protein (n = 3). The rate of degradation of I-RNA transcripts was thus at least 25-fold greater than that of the G-RNAs. After several hours of incubation with an excess of I-RNase, G-RNAs were also substantially degraded by I-RNase (data not shown). This could indicate that I-RNase does not have an absolute specificity for I-RNA. Alternatively, the degradation of G-RNA could reflect low levels of other nucleases in the I-RNase preparation. The degradation of I-RNA transcripts was seen to be dependent on the presence of $MgCl_2$ and did not require ATP (Figure 1C).

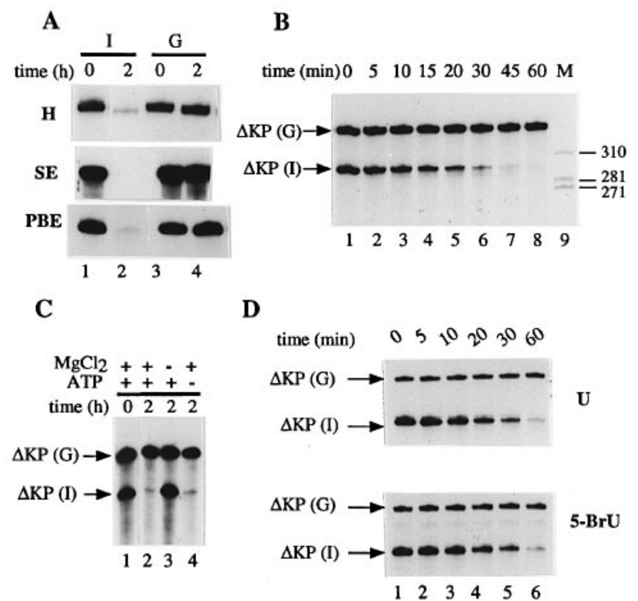

Fig. 1. Specific degradation of I-RNA. (**A**) Samples of ΔKP I-RNA and G-RNA were incubated for 0 or 2 h in HeLa cell nuclear extract (H), sheep uterine smooth muscle extract (SE) and pig brain extract (PBE, partially purified). All three extracts exhibited I-RNase activity. (**B**) Time course of the degradation of ΔKP I-RNA and G-RNA transcripts. The two RNAs were incubated in the same tubes with pig brain I-RNase for the times indicated. The G-RNA remained intact over a time course in which the I-RNA was degraded completely. (**C**) I-RNA and G-RNA were incubated for 2 h under standard conditions (lane 2) or in the absence of added $MgCl_2$ (lane 3) or ATP (lane 4). I-RNase activity was dependent on Mg^{2+} but not ATP. (**D**) A time course of degradation of ΔKP I-RNA and G-RNA transcripts which contain either uridine (U) or 5-bromouridine (5-BrU). The transcripts were incubated in the same tube with I-RNase for the times indicated. Quantitation by phosphorimaging verified that the rate of degradation of the ΔKP I-RNA transcript was identical in the absence or presence of 5-BrU. These data show that I-RNase does not specifically target RNAs containing 5-BrU, but rather is specific for I-RNA.

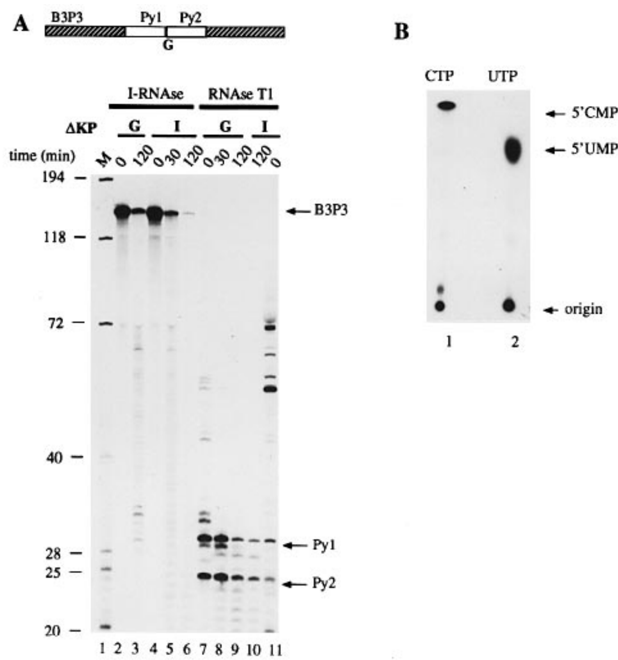

Fig. 2. Non-specific cleavage by I-RNase. (**A**) The RNA transcript B3P3 was used to assess the specificity of the site of cleavage by I-RNase. The transcript is shown schematically; it contains two blocks of pyrimidine residues separated by a single guanosine (or inosine). B3P3 RNA containing either G (lanes 2, 3, 7, 8 and 9) or I (lanes 4, 5, 6, 10 and 11) was incubated with either I-RNase (lanes 7–11) or RNase T1 (lanes 2–6). Two degradation products (24 and 30 nucleotides) corresponding to the two pyrimidine blocks in the transcript are obvious after cleavage by RNase T1, but not by I-RNase. Therefore, I-RNase, unlike RNase T1, does not appear to cleave specifically at inosine residues. (**B**) TLC analysis of degradation products after incubation of ΔKP I-RNA transcripts synthesized using either [α-^{32}P]UTP (lane 1) or [α-^{32}P]CTP (lane 2). The solvent used in this analysis was sodium formate, pH 3.4, which separates individual mononucleotides. The presence of only a single spot from either [^{32}P]UTP- or [^{32}P]CTP-labelled transcripts, corresponding to either 5′-UMP or 5′-CMP respectively, as determined from unlabelled standards, indicates that degradation of I-RNA by I-RNase yields 5′-NMPs.

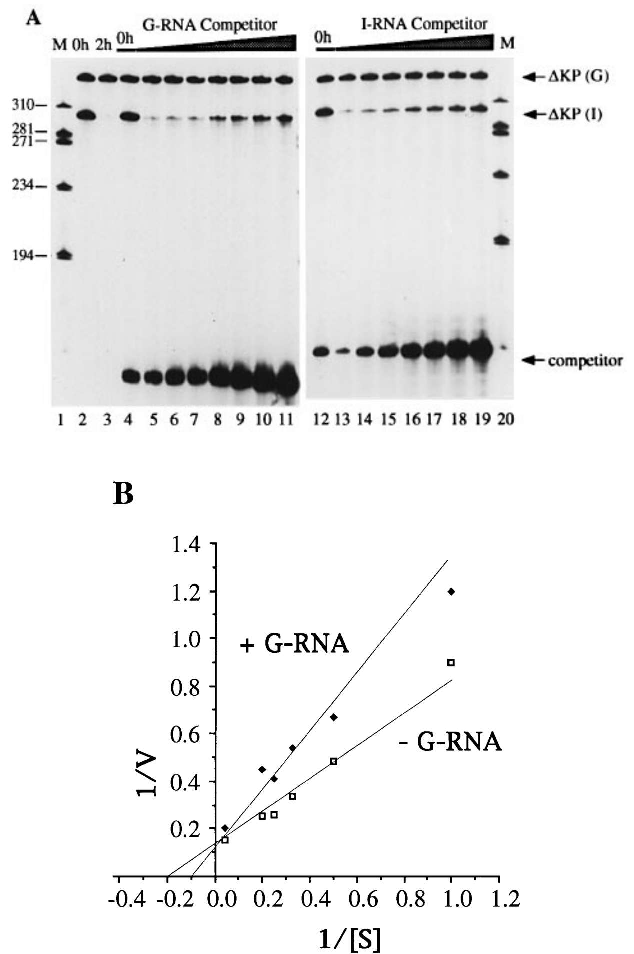

Fig. 3. Preferential binding to I-RNA. (**A**) I-RNase assays were carried out for 0 or 2 h using 5 ng (50 fmol) of ΔKP I-RNA and G-RNA substrates. Assays were carried out in the absence of competitor RNA (0 and 2 h, lanes 2 and 3) or with increasing concentrations of ΔSL G-RNA competitor (60-, 60-, 120-, 180-, 240-, 300-, 360- and 480-fold molar excess, lanes 4–11) or I-RNA competitor (40-, 40-, 80-, 120-, 160-, 200-, 240- and 320-fold molar excess, lanes 12–19). The competitor RNAs, which were labelled to lower specific activity, can be seen at the bottom of the gel. Lanes 4 and 12 show undigested samples containing the same concentration of competitor as lanes 5 and 13. Note that the I-RNA competitor is itself digested (compare lanes 12 and 13). The remaining lanes are 2 h digestions, except for lane 'M' which contains ϕX *Hae*III markers. (**B**) A Lineweaver–Burke plot of I-RNase activity assayed by decrease of acid-precipitable counts. G-RNA acts as a purely competitive inhibitor of I-RNase as shown by the lack of effect upon V_{max} but the increase in the apparent K_m in the presence of 1.2 μM G-RNA competitor (+G-RNA).

We carried out a number of experiments to investigate the substrate specificity of the I-RNase. Firstly, we wanted to establish whether the novel activity was specific for I-RNAs or if it recognized and degraded RNAs containing any unconventional nucleotides. To this end, ΔKP transcripts were synthesized in which UTP was substituted by 5-bromo UTP (5-BrU) in addition to containing either G or I. As I and 5-BrU were being substituted for G and U residues in the ΔKP transcripts, respectively, the number of modified nucleotides incorporated was different in each case. While the ΔKP transcripts contained 100 I or 112 G residues, only 41 or 57 5-BrU residues were included in the I- and G-RNAs, respectively. Therefore, a direct comparison of the effect of an equivalent number of modified residues in an RNA transcript could not be made. Nevertheless, a sufficient number of 5-BrU residues were present in the modified RNA to make a qualitative assessment of its effect on degradation. RNA transcripts which contain <41 I residues (e.g. pBluescript II SK I-RNA) were still degraded preferentially by I-RNase (see Figure 4, lanes 1 and 2). Degradation of ΔKP transcripts containing either U or 5-BrU in addition to I or G were thus analysed over a time course from 0 to 60 min (Figure 1D). Quantitation by phosphorimaging showed that there was no consistent difference in the rate of degradation of 5-BrU- and U-containing RNA. In the experiment of Figure 1D, the rates were indistinguishable (462 ng RNA/min/mg for I-RNA and 19 ng RNA/min/mg for G-RNA). This observation demonstrated that I-RNase is specific for inosine rather than for any unconventional base. Moreover, this demonstrates that I-RNase is not inhibited by the presence of other anomalous nucleotides.

I-RNase does not cleave at specific sites

The specificity of I-RNase could arise either from preferential binding of the enzyme to I-RNA with subsequent non-specific degradation, or from a high specificity for cleavage

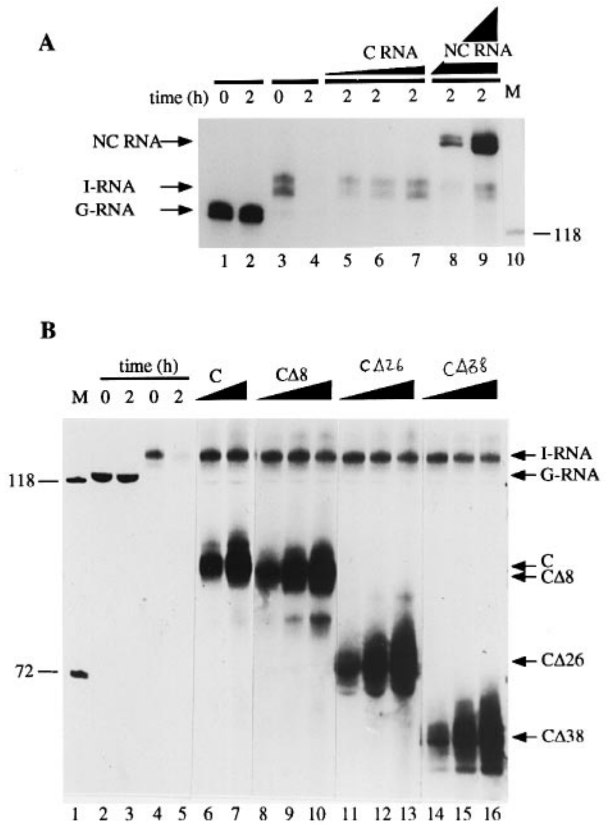

Fig. 4. I-RNase degrades single-stranded I-RNA. (**A**) Lanes 1 and 2 show pBSK$^+$ G-RNA present at 0 and 2 h after incubation with I-RNase. Lanes 3 and 4 show pBSK$^+$ I-RNA at 0 and 2 h. In all lanes, 2.5 ng of substrate RNA were used. Lanes 5–7 show the effects of adding 1.3-, 2.5- and 6.3-fold molar excesses of a complementary pBSK$^+$ G-RNA (C RNA) (lane 5, 2.5 ng: lane 6, 5 ng: lane 7, 12.5 ng). In lanes 8 and 9, a 22- and 85-fold molar excess of a non-complementary RNA competitor (NC RNA) were added. Significant protection of I-RNA from degradation was seen with all concentrations of the C RNA, but only with the 85-fold excess of NC RNA. (**B**) Protection of I-RNA in single-stranded bulges. I-RNA was synthesized from pBSK$^+$ polylinker and 5 ng was digested alone (lanes 3 and 4) or in the presence of complementary RNAs (lanes 6–16). The complemenary RNAs were either full-length pBSK$^+$ transcripts, or pBSK$^+$ transcripts which contain deletions of 8, 26 or 38 nucleotides (CΔ8, CΔ26 and CΔ38 respectively). Thus, the hybrid RNAs either contain perfect duplexes (C, lanes 6 and 7) or duplexes with I-RNA single-stranded bulges of 8, 26 or 38 nucleotides (lanes 8–16). Lanes 2 and 3 show the sense G-RNA at 0 and 2 h respectively. Lanes 4 and 5 show the sense I-RNA at 0 and 2 h respectively. Full-length complementary RNAs were added to 10 and 25 ng, lanes 6 and 7 (6.3- and 13-fold molar excess). CΔ8, CΔ26 and CΔ38 were added to 10, 25 and 50 ng each in lanes 8–10, 11–13 and 14–16 respectively. These represent molar excesses of: CΔ8, 3-, 7- and 14-fold; CΔ26, 3.5-, 9- and 18-fold; CΔ38, 4.5-, 11- and 21.5-fold. Full protection of the I-RNA was observed in the presence of the lowest concentration of each complementary RNA (lanes 6, 8, 11 and 14). This demonstrates that I-RNA in single-stranded bulges is not a substrate for I-RNase.

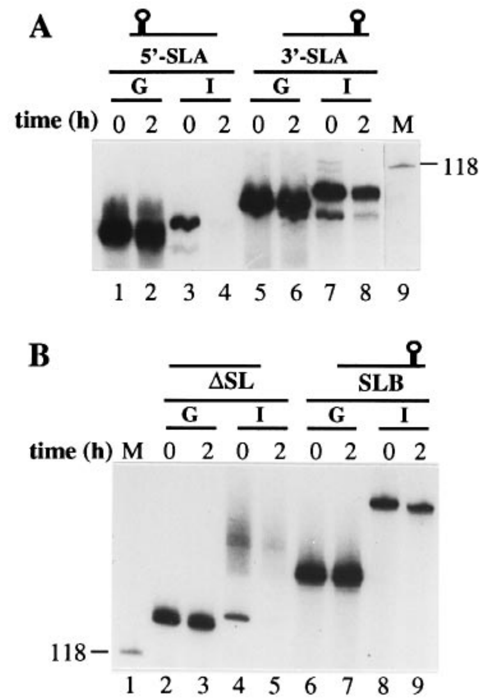

Fig. 5. I-RNase is a 3′→5′ exonuclease. (**A**) RNAs were synthesized with a hairpin at the 5′ or 3′ end by transcribing the template SLA using either SP6 or T7 RNA polymerase. Lanes 1 and 2 show the G-RNA with the 5′ hairpin at 0 and 2 h, respectively, and lanes 5 and 6 show the G-RNA with the 3′ hairpin at 0 and 2 h. Similarly, lanes 3 and 4 show the I-RNA with the 5′ hairpin at 0 and 2 h, respectively, and lanes 7 and 8 show the I-RNA with the 3′ hairpin at 0 and 2 h. Only the I-RNA with the 5′ hairpin is degraded at 2 h. (**B**) The transcript ΔSL is unstructured while transcript SLB has a hairpin at the 3′ end. Lanes 2 and 3 show the linear G-RNA at 0 and 2 h, respectively, and lanes 6 and 7 show the G-RNA with the 3′ hairpin at 0 and 2 h. Similarly, lanes 4 and 5 show the linear I-RNA at 0 and 2 h, respectively, and lanes 8 and 9 show the I-RNA with the 3′ hairpin at 0 and 2 h. Only the linear I-RNA is degraded at 2 h. Thus 3′ hairpins block I-RNase consistent with a 3′→5′ mode of action.

adjacent to I residues. The latter case would be analogous to RNase T1 which specifically cleaves to the 3′ side of G residues leaving a 3′ phosphate group. To address the question of whether I-RNase cleaves only adjacent to I residues, we digested the transcript B3P3 with I-RNase (Figure 2A). B3P3 contains two blocks of 24 pyrimidine residues separated by a single G (or I) residue (Mullen *et al.*, 1991). If I-RNase cleaved only at I residues, two major cleavage products of 25 and 29 nucleotides would be detected, corresponding to the two pyrimidine blocks. As a control, cleavage was also carried out with RNase T1 which cleaves after G residues and with somewhat reduced kinetics at I residues (Steyaert *et al.*, 1991). Cleavage of B3P3 RNA by RNase T1 yielded two major bands of 25 and 29, as expected (Figure 2A). Note that RNase T1 digestion was almost complete within the time that it took to quench the ‘zero’ time reactions (Figure 6A, lanes 7 and 11). In contrast, cleavage of B3P3 I-RNA by I-RNase yielded no specific cleavage products at any time, but instead gave rise to a faint ladder of degradation products. Similar results were obtained when a different pyrimidine-rich transcript was used for analysis. These data demonstrate that I-RNase does not cleave specifically at I residues, but rather suggest that it may cleave at every phosphodiester bond.

We next analysed the products of complete digestion by I-RNase. Analysis of I-RNase-digested ΔKP I-RNA was carried out by thin layer chromatography (TLC). ΔKP I-RNA transcripts were synthesized using either [α-^{32}P]UTP or [α-^{32}P]CTP. If cleavage by I-RNase produces 3′-NMPs, the label would be transferred to all nucleotides adjacent to U or C residues, resulting in a mixture of the four labelled 3′-NMPs. In contrast, cleavage

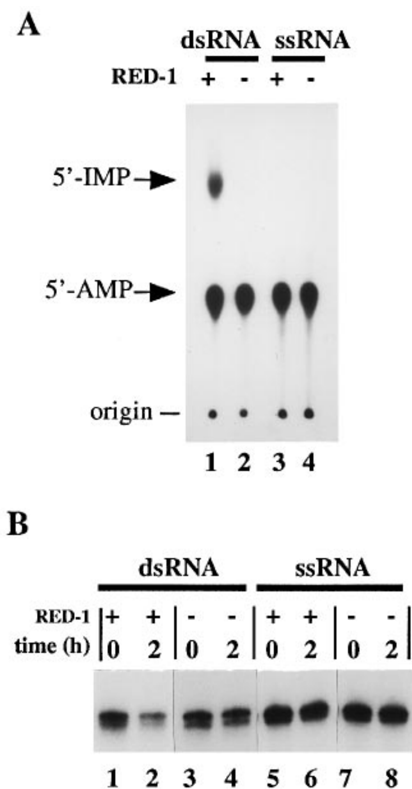

Fig. 6. The products of RED-1 deamination are degraded by I-RNase. (**A**) T3-transcribed pBluescript RNA labelled with [α-^{32}P]ATP was incubated in the absence or presence of RED-1, either alone (ssRNA) or after annealing to a complementary T7 transcribed RNA (dsRNA). Following RED-1 treatment, the RNAs were digested with RNase P1, and the digestion products then fractionated by TLC. Adenosine residues were only converted to inosine in the dsRNA incubated with RED-1 (lane 1). (**B**) Double- or single-stranded Bluescript RNAs treated in the absence or presence of RED-1 were incubated subsequently with I-RNase under standard conditions. Only pBluescript dsRNA which had been pre-treated with RED-1 was degraded after 2 h incubation with I-RNase (lane 2). Unmodified RNAs (ssRNA or dsRNA without RED-1 treatment) remained undegraded at 2 h (lanes 4, 6 and 8). The autoradiograph shows the T3-transcribed RNA strand. Similar results were obtained when the T7 strand was visualized.

yielding 5′-NMPs would result in the label remaining associated only with UMP or CMP respectively. As shown in Figure 2B, a single [^{32}P]NMP spot was detected upon digestion of each I-RNA substrate. The major spot resulting from digestion of either [^{32}P]UTP- or [^{32}P]CTP-labelled I-RNA corresponds to the generation of 5′-UMP or 5′-CMP respectively, as determined by the position of unlabelled standards. The spot detected at the origin in both lanes probably represents a small proportion of incompletely digested oligoribonucleotides since its relative intensity diminished when increasing amounts of I-RNase were used to digest the transcripts (data not shown). Therefore, I-RNase appears to digest I-RNA completely, yielding 5′-NMPs.

Preferential binding to I-RNA

The preceding data demonstrate that the specificity of I-RNase does not result from cleavage only adjacent to I residues. An alternative explanation for I-RNase specificity is that the enzyme binds preferentially to I-RNA. To investigate this possibility, competitor RNAs were mixed with the ΔKP I- and G-RNA substrates prior to incubation with I-RNase. If the competitor RNA is able to bind to I-RNase it should cause a decrease in the rate of degradation of the test I-RNA. Both I-RNA and G-RNA competitor RNAs were tested over a range of molar excesses (Figure 3A, lanes 5–11 and 13–19). Both RNAs competed the I-RNase degradation of the target ΔKP I-RNA transcript and the I-RNA competitor appeared to be a more effective competitor (compare lanes 5–7 with 13–15). However, this analysis underestimates the competition by I-RNA since the competitor itself is digested. This can be seen by comparing the amount of competitor at 0 h in lanes 4 and 12 with the amount remaining after 2 h incubation in lanes 5 and 13. While the G-RNA competitor remains intact, the I-RNA competitor is largely degraded. Note that the competitor RNAs were labelled to much lower specific activities. Thus, these data demonstrate that I-RNase binds both G-RNA and I-RNA and suggest that I-RNA is bound with higher affinity. Interestingly, we found that poly(I), poly(G) and yeast tRNA (seven species of which contain inosine) were all much less effective at competition than the mixed composition RNAs shown in Figure 3A (data not shown).

To obtain a more rigorous estimate of the difference in the ability of I- or G-RNA to bind to I-RNase, we performed simple Michaelis–Menten kinetic analysis and determined the K_m of I-RNase for I-RNA and the K_i for G-RNA. The initial rate of degradation of ΔKP I-RNA at various concentrations was determined for a constant concentration of I-RNase by measuring the decrease in acid-precipitable radioactivity. Since I-RNase is an exonuclease (see below), the loss of precipitable counts should be proportional to the breakage of phosphodiester bonds; this condition would not necessarily hold for endonucleases because initial cleavage events might yield fragments that are still fully precipitable. The K_m for ΔKP I-RNA was determined subsequently by Lineweaver–Burke plots (Figure 3B) to be $9.0 \pm 4.4 \times 10^{-9}$ M ($n = 4$). The rate of degradation was then assessed under the same conditions, but in the presence of a fixed concentration of one of two different G-RNA competitors (ΔSL or ΔKP). Analysis of these data indicated that G-RNA acted as a purely competitive inhibitor, with no change in the V_{max} of the enzyme but a decrease in the apparent K_m (Figure 3B). In duplicate assays, the K_i of I-RNase for ΔSL G-RNA was determined to be 1.5×10^{-6} M and 1.2×10^{-6} M (K_m is decreased by a factor of $1+[I]/K_i$ where [I] is the concentration of inhibitor). The K_i for the ΔKP G-RNA competitor was similarly determined to be 1.2×10^{-6} M. Assuming that K_m and K_i provide a reasonable estimate of the dissociation constants for I-RNA and G-RNA, these data suggest that the binding of G-RNA to I-RNase is ~300-fold weaker than the binding of I-RNA. It is worth recalling that K_m is only a good estimate of the dissociation constant, K_D, if the rate of subsequent catalytic steps are slow compared with the dissociation rate for the enzyme–substrate complex (see Fersht, 1985). Given that it is unlikely that the rate constant of these steps for G-RNA is greater than for I-RNA, the apparent 300-fold difference in affinities is likely to be a conservative estimate. Thus, preferential binding of I-RNA to I-RNase is probably sufficient to explain the high specificity for degradation of I-RNA.

I-RNase does not degrade dsRNA

We next investigated the specificity of I-RNase for dsRNA or ssRNA. Complementary RNA transcripts were synthesized using the polylinker of pBluescript SK^+ (Stratagene) as a template. One strand was labelled with [^{32}P]UTP and contained guanosine or inosine, while the complementary transcripts contained guanosine and were unlabelled. The labelled pBluescript SK I-RNA, but not the G-RNA, was degraded by I-RNase, as expected (Figure 4A, lanes 1–4). However, if it was first annealed with the complementary RNA strand (see Materials and methods for annealing conditions), I-RNA degradation was largely inhibited (Figure 4A, lanes 5–7). Addition of a 1- to 5-fold excess of complementary RNA led to protection from degradation (compare lanes 5–7 with lane 4). That this inhibition of degradation was due to base pairing rather than competitive inhibition by binding of the complementary RNA to I-RNase was confirmed by the much higher concentrations of an unrelated non-complementary RNA competitor that were required to achieve similar levels of inhibition (lanes 8 and 9; 25- and 100-fold excess respectively). These observations demonstrate that I-RNase does not degrade fully base-paired I-RNA.

We next tested whether I-RNase is able to degrade I-RNA which is largely double-stranded, but which contains unpaired single-stranded regions of 8, 26 or 38 nucleotides containing two, nine or 10 I residues respectively. These bulged RNA duplexes were created by annealing a full-length pBluescript SK I-RNA transcript with a complementary pBluescript SK G-RNA transcript containing an internal deletion in the polylinker. No degradation of any of the test substrates by I-RNase was detectable (Figure 4B). Since we know that I-RNase can degrade short single-stranded I-RNAs of ~30 nucleotides (data not shown), but not fully base-paired RNA (Figure 4A), this observation suggests that I-RNase is an exonuclease and that the inosine-containing single-stranded regions of these substrates are protected from degradation by the flanking double-stranded regions.

I-RNase is a 3′→5′ exonuclease

Exonucleases degrade nucleic acids in either a 3′→5′ or a 5′→3′ direction. To determine the polarity of I-RNase, we have created transcripts containing a stable hairpin at either the 5′ or 3′ end. Based upon the preceding observations, if I-RNase is a 5′→3′ exonuclease, degradation would be blocked by the presence of a hairpin at the 5′ end of the I-RNA substrate but not when the stem–loop is at the 3′ end. Conversely, if I-RNase functions as a 3′→5′ exonuclease, its activity would be blocked by a stem–loop located at the 3′ but not the 5′ end of the substrate. Uncapped I- and G-RNA transcripts were synthesized from the template pSLA using either the SP6 or the T7 promoter (transcripts were initiated with 5′-GMP, refer to Materials and methods). This generated complementary transcripts which had a stem–loop at either the 5′ or 3′ end, respectively. Upon incubation with I-RNase, the I-RNA transcript with the stem–loop at the 5′ end was degraded (Figure 5A, lane 4) while the transcript with a 3′ hairpin remained intact (lane 8). This observation, which was made consistently in four identical assays, suggests that I-RNase is a 3′→5′ exonuclease. To confirm this conclusion, degradation of a second pair of transcripts was tested (in triplicate). I- and G-RNA transcripts were synthesized from the constructs pΔSL and pSLB. The SLB and ΔSL transcripts were identical except that SLB transcripts had a stable hairpin at the extreme 3′ end. Only the I-RNA without a 3′ hairpin was degraded significantly (Figure 5B, compare lanes 5 and 9). The data shown were obtained with uncapped I- and G-RNA; identical data were obtained when the RNAs were capped. Taken together, these data confirm that I-RNase is a 3′→5′ exonuclease.

I-RNase degrades the products of RED-1 deamination

One instance in which inosine occurs naturally in cells is as the result of deamination of dsRNAs by enzymes such as dsRAD (Hurst *et al.*, 1995; Dabiri *et al.*, 1996; Maas *et al.*, 1996) or RED-1 (Melcher *et al.*, 1996). The inosine-containing products of deamination may be substrates for degradation by I-RNase. The previous experiments were carried out on RNAs in which I had been incorporated in place of G by bacteriophage RNA polymerases (Figures 1–5). We next tested whether I-RNase could specifically degrade I-RNAs in which inosine was introduced by the more physiologically relevant mechanism of RED-1 modification.

Complementary RNA transcripts labelled with [α-^{32}P]ATP were synthesized using the template pBluescript SK, from either the T3 or T7 promoters. The RNAs were then annealed, and the dsRNA treated with recombinant RED-1. In order to determine what percentage of the A residues were converted to I, a sample of RED-1-treated RNA was digested with RNase P1 and then analysed by TLC under conditions where the spots corresponding to 5′-AMP and 5′-IMP were separated. Inosine was only detected in dsRNA which had been treated with RED-1 (Figure 6A, lane 1). As expected, untreated dsRNA and ssRNA incubated in the presence or absence of RED-1 remained unmodified (lanes 2, 3 and 4, respectively). Quantitation of the double-stranded pBluescript RNA deamination by phosphorimaging revealed that 13.6 ± 0.5% ($n = 3$) of A residues were converted to I. Allowing for the adenine content of the double- and single-stranded regions, this corresponds to 17.6% conversion of adenosines in dsRNA, which is about a third of the maximum obtainable value with dsRAD (Polson and Bass, 1994). This in turn corresponds to the introduction of an average of 3.2 I residues into the T3-synthesized RNA strand and 2.8 into the T7 strand if all adenines in dsRNA are equally susceptible to deamination (which strictly is not the case, Polson and Bass, 1994). Following deamination of the double-stranded pBluescript RNA by RED-1, the modified RNA was treated with I-RNase under standard conditions. Prior to incubation of the modified double-stranded pBluescript RNA with I-RNase, the duplex was disrupted by heat denaturation. The results showed that the RNAs which contain inosine as the result of deamination by RED-1 were degraded selectively by I-RNase (Figure 6B, compare lanes 1 and 2). Quantitation of the data of Figure 6B by phosphorimaging showed that ~50% of the initial amount of RNA remained at time 2 h when the RNA had been treated with RED-1 followed by I-RNase (compare lanes 1 and 2). In contrast, dsRNA incubated under the same conditions in the absence of RED-1 and then with

I-RNase was undegraded at 2 h (compare lanes 3 and 4). ssRNA (T3 strand) pre-incubated in the presence or absence of RED-1 was not degraded by I-RNase (lanes 5–8). In the absence of denaturation of the RNAs prior to incubation with I-RNase, no degradation was observed under the assay conditions used (data not shown). Neither was any degradation of ΔKP G-RNA observed following heat denaturation and I-RNase treatment under identical conditions. These results have been reproduced qualitatively three times, although the data has not been quantitated on each occasion.

These data therefore demonstrate that RNAs with as few as 3–4 I residues introduced by RED-1 become specific substrates for I-RNase upon denaturation. Moreover, they demonstrate that the preferential degradation of I-RNA is due to the presence of inosine rather than to the absence of guanosine; this distinction could not be made formally from the previous data. The data are consistent with I-RNase playing a role in degradation of deaminated RNA in the cell, although modification to the levels observed here does not destabilize the dsRNA sufficiently to allow direct access of I-RNase without prior denaturation (see Discussion).

Discussion

In this study we have characterized a novel ribonuclease, I-RNase, which is specific for inosine-containing ssRNA, which is divalent cation dependent, and which acts as a 3′→5′ exonuclease yielding 5′-NMPs. The most interesting of these properties is the novel specificity for I-RNA. I-RNase preferentially degraded RNA that contained inosine as a result of incorporation by bacteriophage polymerases during *in vitro* transcription (Figures 1–5), and also by the more physiologically relevant route of modification by the RED-1 deaminase (Figure 6). The basis of I-RNase specificity is not due to exclusive or preferential cleavage adjacent to I residues (Figure 2). Rather, competition experiments (Figure 3) indicated that the specificity arises at the level of binding to RNA. This was not immediately obvious from the standard analysis involving gel electrophoresis and autoradiography of samples (Figure 3A). However, a more rigorous kinetic analysis indicated that G-RNA behaved as a purely competitive inhibitor of I-RNA degradation by I-RNase. The determined K_i for G-RNA (~1.3×10^{-6} M) was some ~300-fold higher than the K_m of the enzyme for I-RNA. Therefore, assuming that these constants provide a reasonable estimate of the binding affinity (see Results section above), preferential binding to I-RNA appears to provide the basis for the specificity of the enzyme. The specificty of I-RNase is for RNA that contains inosine rather than for RNA that lacks guanosine, as demonstrated by its ability to specifically degrade RED-1-modified RNA (Figure 6). Finally, the specificity of the enzyme appears to be for inosine, rather than for any non-conventional bases, as indicated by the stability of G-RNA containing 5-BrU (Figure 1D). It might be expected that naturally occurring uridine analogues such as pseudouridine and dihydrouridine would also not be targeted by I-RNase. It is worth noting that although I-RNase activity is novel, it is possible that the enzyme is a previously characterized nuclease that hitherto has not been tested with I-RNA substrates.

Biological substrates of I-RNase?

Given the novel specificity of I-RNase, what might be its natural substrates? The properties of I-RNase that we have characterized allow some informed speculation between possible substrates. Inosine commonly occurs in cellular RNAs as a result of three processes. The first is in the wobble position of the anticodon loop of some tRNAs. This was thought originally to occur via insertion of the base hypoxanthine (Elliot and Trewyn, 1984), but has been shown more recently to involve hydrolytic deamination of adenine (Auxilien *et al.*, 1996). We have shown that single-stranded regions containing up to 10 inosines flanked by dsRNA stems are not degraded (Figure 4B). Therefore, the single inosine within the anticodon loop of tRNAs is unlikely to be a target. Consistent with this, we found that yeast tRNAs were particularly weak competitors of I-RNase (data not shown). The second process which introduces inosine into RNA is specific editing of some cellular RNAs by deamination of A to I. The A to I conversions at specific positions of the GluRB mRNA (and probably other cellular RNAs) lead to changes in the coding capacity of the mRNA because the I residues in the mRNA are decoded as G rather than A. This editing is most likely carried out by the RED-1 enzyme (Melcher *et al.*, 1996) and/or dsRAD (Hurst *et al.*, 1995; Dabiri *et al.*, 1996; Maas *et al.*, 1996) and relies upon localized secondary structure within the exons which is probably sufficiently destabilized by the specific editing event to prevent subsequent modifications (Higuchi *et al.*, 1993; Maas *et al.*, 1996). Specifically edited mRNAs with a limited number of inosines also seem unlikely to be I-RNase targets for two reasons. In GluRB mRNAs the edited positions are distant from the 3′ end; the 3′-untranslated region alone is 2.7 kb (Koehler *et al.*, 1994). Given that I-RNase is a 3′→5′ exonuclease, it is likely that specific degradation requires I residues to lie within proximity to the 3′ end. It is possible that the poly(A) tail may in itself be sufficient to protect many edited mRNAs either because it is a region devoid of inosines and/or because poly(A)-binding proteins would block entry of 3′→5′ exonucleases. We are planning to investigate this issue. Second, compartmentation could conceivably protect edited mRNAs. We have only detected I-RNase in nuclear fractions, but our inability to detect I-RNase in the cytoplasmic S-100 fractions was due to the high levels of general nuclease activity. Therefore, while we cannot rule out cytoplasmic I-RNase activity, the possibility remains that it could be restricted to the nucleus. In this case, edited mRNAs that are transported to the cytoplasm efficiently would no longer be vulnerable to I-RNase attack. Thus, tRNAs and edited mRNAs, which have specific functions in the cell, are not probable targets for I-RNase.

The third source of I in RNA, related to specific editing, is the wholesale conversion of A to I within extended regions of dsRNA as a result of dsRAD activity (Bass and Weintraub, 1988; Wagner *et al.*, 1989). The resultant I–U base pairs are less stable than the original A–U base pairs, causing the RNA to become less double-stranded in character. dsRAD was originally characterized in *Xenopus laevis* oocytes as an activity that caused irreversible unwinding of dsRNA, and subsequently has been identified in a number of organisms throughout the animal kingdom

(Wagner *et al.*, 1990; reviewed in Bass, 1993). The binding of dsRAD to dsRNA does not appear to be sequence specific, although in short RNA duplexes there is some selectivity in the choice of modification sites (Polson and Bass, 1994). In long dsRNA duplexes (>100 bp) modification is more efficient, and up to 50% of adenosines can be modified in either strand of the duplex (Nishikura *et al.*, 1991; Polson and Bass, 1994).

While dsRAD and its related isoform RED-1 have been implicated in specific RNA editing of cellular GluRB mRNA (Hurst *et al.*, 1995; Dabiri *et al.*, 1996; Maas *et al.*, 1996; Melcher *et al.*, 1996) and hepatitis delta virus RNA (Polson *et al.*, 1996), it is likely that one or both of these enzymes may also have other roles. One of the earliest suggestions, due to the targeting of dsRNA, was a role in antiviral defence (Bass and Weintraub, 1988). The demonstration of induction of dsRAD expression by interferon is highly supportive of such a role (Patterson and Samuel, 1995; Patterson *et al.*, 1995). In addition, the presence of A→G and U→C hypermutations of viral RNAs during persistent infection observed with some negative-stranded RNA viruses is consistent with dsRAD acting upon viral dsRNA intermediates (Bass *et al.*, 1989; Cattaneo and Billeter, 1992). The I-RNase activity, like dsRAD, appears to be widespread (readily detectable in HeLa cells, smooth muscle and brain) consistent with a general role rather than a cell type-specific function. Moreover, the enzyme appears to be more active on larger RNAs with multiple I residues (compare Figure 6B with previous figures). We therefore speculate that I-RNase may have a general role in cellular antiviral defence by degrading RNAs that have been 'tagged' by wholesale A→I modifications. Although dsRAD is capable of destroying the integrity of viral open reading frames, such RNAs potentially could still compete with cellular RNAs for the translation machinery. Efficient degradation of such RNAs would give the cell a better chance of combating the viral attack.

Consistent with this speculation, we showed that I-RNase was able to degrade RNA that had first been modified by RED-1 (Figure 6). Nevertheless, we found that under the conditions of our experiment, the modified dsRNA had to be denatured first to allow subsequent degradation by I-RNase. Taken at face value, this suggests that the immediate products of deamination of dsRNA are not substrates for I-RNase. One possible explanation is that I-RNase degrades heavily deaminated RNA inside the cell, but that some other enzymatic activity such as an RNA helicase is necessary to present the modified RNA in recognizable form. The coupled action of an RNA helicase with a 3′→5′ exonuclease has been demonstrated in the *Escherichia coli* degradosome (Py *et al.*, 1996). Moreover, the conditions of the RED-1/I-RNase experiment in Figure 6 were not ideal. First, the 17.6% A→I conversion was well below the maximal obtainable, and so the double-stranded region was not fully destabilized. Probably, this could be improved by using longer RNA duplexes which are more efficient deaminase substrates. It is possible that by increasing the level of A→I modification the helix may be sufficiently destabilized that heat denaturation of the RNA is unnecessary prior to degradation by I-RNase. Moreover, other specific features of the Bluescript polylinker duplex made it a suboptimal substrate for this experiment. The RNA duplex at the 3′ end of the T3 strand was closed by a G–C base pair followed by two A–U base pairs and then 13 G–C base pairs. Given that adenines at the 3′ end of the duplex are poor substrates for dsRAD (Polson and Bass, 1994) and so are probably not modified, the 3′ end of this duplex would be expected to stay fully double stranded and so resistant to I-RNase unless first denatured. Likewise, a 9 bp GC stretch towards the other end of the duplex probably protected the 3′ end of the T7 strand from I-RNase attack. Nevertheless, even under less than optimal conditions, the modified Bluescript RNA was a substrate for I-RNase. We are planning to test whether other dsRNAs can be degraded directly by I-RNase after modification by RED-1 or other members of the dsRAD family.

Enzymes that are involved in antiviral pathways are often inducible by interferon and/or virus infection. We have attempted to detect changes in the levels of I-RNase activity in interferon-treated cells. However, we have found that in unfractionated cell extracts the background general nuclease activities are too high to allow changes in I-RNase activity to be discerned. However, it is possible that the enzyme could participate in an inducible pathway without being induced itself. One further speculation that can be made is that the proposed dsRAD/I-RNase pathway could also mediate antisense-mediated inhibition of gene expression (Nellen and Lichtenstein, 1993).

In conclusion, we have characterized an RNase whose novel specificity suggests a role in cellular antiviral defence. The elucidation of the precise biological substrate specificity of I-RNase and its role and localization in the cell will be greatly facilitated by its purification to homogeneity and molecular cloning. We are currently pursuing this goal.

Materials and methods

Constructs and transcripts

Constructs used as templates for *in vitro* transcription or for transfection were all prepared by standard cloning procedures (Sambrook *et al.*, 1989). The construct ΔKP comprises spliced exons 2 and 3 of α-TM, generated by deletion between the *Kpn*I and *Pvu*II sites at the ends of the intron in construct pGC+DX (Smith *et al.*, 1993). The construct B3P3 contains the branch point and polypyrimidine tract of α-TM exon 3 between the *Sma*I and *Acc*I sites cloned into the *Sma*I site of pGEM 4 (Mullen *et al.*, 1991). The construct pBluescript SK was used to synthesize complementary RNAs to investigate the ability of I-RNase to degrade dsRNAs. The T3 promoter was used for the synthesis of transcripts containing I or G when pBluescript SK was linearized with *Asp*718. The T7 promoter was used to synthesize complementary RNAs which contained G when pBluescript SK was linearized with *Xba*I. To make dsRNAs, the complementary RNAs were mixed together in TE buffer and then heated at 70°C for 5 min. The RNAs were cooled slowly to 30°C (60–90 min in a heating block). The constructs pBluescript SK Δ8, pBluescript SK Δ26 and pBluescript SK Δ38 were made by deleting the polylinker sequence of pBluescript SK between the *Xba*I site and the *Bam*HI, *Eco*RI and *Hin*dIII restriction sites respectively. Each of these plasmids were linearized with *Not*I, and the transcripts were synthesized using the T7 promoter. The construct pSLA was made by inserting the hairpin-forming sequence 5′ $GGG(CGG)_6$ GAATT$C(CCG)_6$ CCC 3′ into the *Pvu*II site of pSP70 (this is the construct referred to as HP in Liu *et al.*, 1996). The complementary sequences which anneal to form the stem are underlined. pSLA was linearized with *Bgl*II, and the SP6 promoter was used to synthesize the transcript 5′-SLA. To synthesize the transcript 3′-SLA, pSLA was linearized with *Xho*I, and the T7 promoter was used for transcription. Both of these transcripts were synthesized using 5′-GMP to prime transcription. The construct ΔSL was used to transcribe linear RNAs from the T7 promoter with the

sequence: GpppGGGAGACAAGCUUGAGCUGGAUGCCGCCUCU-GCUGCUGCGCACAUUUCAUUUAUAUUCUGUCCUUUCCCCUU-UUUCUCCCUCUUCUUUACCUCCUCCCCUUUGGUUGGAGGUGG-GUGGGUGAGAAUU. The construct pSLB was used to synthesize RNAs, using the T7 promoter, which contained a sequence at the 3′ end which forms a stable stem–loop, as follows: GpppGGGAGACAA-GCUUGAGCUGGAUGCCGCCUCUGCUGCUGCGCACAUUUCAU-UUAUAUUCUGUCCUUUCCCCUUUUUCUCCCUCUUCUUUACCU-CCUCCCCUUUGGUUGGAGGUGGGUGGGUGAGAAUUGCUUG-CAAUUCUCACCCACCCAGAAUU. The sequence which anneals to form the stem is underlined; the loop has the sequence of a stable tetraloop. The identity of all constructs was confirmed by sequencing.

Extract preparations and partial purification of I-RNase

HeLa cell nuclear extracts were prepared using the modifications of Abmayr *et al.* (1988). Pig brain extract was prepared as follows: 100 g of pig brain was homogenized in 200 ml of a low salt buffer [20 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 20 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinin, 2 μg/ml chymostatin, 2 μg/ml leupeptin, 5 mM NaF, and 5 mM β-glycerophosphate], and the salt concentration was then raised to 0.3 M in a total volume of 400 ml. The homogenate was stirred at 4°C for 30 min, centrifuged at 8000 *g* for 20 min, and the supernatant subsequently was centrifuged at 100 000 *g* for 2 h at 4°C. Finally the extract was dialysed against 20 mM HEPES-KOH, pH 7.9.

I-RNase was partially purified from the pig brain extract by three successive chromatography columns: DEAE–Sepharose, CM–cibacron blue and heparin–Sepharose. Each of these columns were run using 20 mM HEPES-KOH, pH 7.9, 50 mM KCl buffer, and the proteins eluted using salt gradients up to 3 M KCl. The column fractions were dialysed against a buffer containing 20 mM HEPES-KOH, pH 7.9, 50 mM KCl for subsequent identification of the fractions containing I-RNase. Activity was assessed using the standard degradation assay.

Sheep uterine smooth muscle tissue extract was prepared by essentially the same method as was used for the pig brain extract; the only exception being that the smooth muscle tissue was ground in liquid nitrogen using a mortar and pestle rather than broken down by homogenization. I-RNase was partially purified from the sheep uterine smooth muscle extract using a heparin–Sepharose column. This column was run using 20 mM HEPES–KOH, pH 7.9, 50 mM KCl buffer, and the proteins were eluted using a salt gradient from 0 to 1 M KCl

In vitro transcription

Standard ^{32}P-labelled RNAs were transcribed from cloning vectors with SP6, T3 or T7 RNA polymerase (Promega) as described previously (Smith and Nadal-Ginard, 1989; Mullen *et al.*, 1991). Unless otherwise stated, transcription was initiated using a $m^7G(5')ppp(5')G$ dinucleotide primer. For uncapped transcripts (SLA, SLB, ΔSL, Figure 5), 5′-GMP was added to the transcription reaction at a final concentration of 2 mM. For synthesis of inosine-containing transcripts, ITP was added in place of GTP at an equivalent concentration. TLC was used to verify the composition of the I-RNA transcripts (Scadden and Smith, 1995). In 50 random transcription reactions, the efficiency of I-RNA transcription ranged from 40 to 80% of the G-RNA transcription efficiency. In the absence of CAP analogue or 5′-GMP, the I-RNA transcription efficiency was reduced further to $<10\%$ of the G-RNA transcription efficency, consistent with the previous report that T7 RNA polymerase can use ITP for transcriptional elongation but that a primer is also required (Milligan and Uhlenbeck, 1989).

Labelled mixed composition competitor RNAs were trace labelled with [α-^{32}P]UTP at a specific activity at least 10-fold lower than substrate RNAs.

Degradation assays

The standard assay for degradation used the same conditions that we use for *in vitro* pre-mRNA splicing. Each 10 μl reaction contained 20–50 fmol of [^{32}P]RNA transcript, 2.5 mM $MgCl_2$, 500 μM ATP, 20 mM creatine phosphate, 2.5 U/μl RNasin, buffer E [12 mM Tris (pH 7.9), 12% (v/v) glycerol, 60 mM KCl, 0.12 mM EDTA, 0.3 mM DTT], and 40–60% extract/fraction. Reactions generally were incubated at 30°C for 0–2 h. Degradation assays were then subjected to proteinase K digestion, phenol/chloroform extraction and ethanol precipitation in the presence of 10 μg of carrier tRNA. Reaction products were analysed by electrophoresis in 8 M urea, 5–10% polyacrylamide gels, followed by autoradiography or phosphorimaging.

For assaying I-RNase activity by loss of acid-precipitable counts, 10 μl reactions (Figure 3B) were assembled according to the standard protocol, using the ΔKP I-RNA. When the incubation at 30°C was complete, the reaction was added to 1 ml of 10% trichloroacetic acid, which additionally contained 10 μg of tRNA. Following incubation on ice for 10 min, the samples were filtered onto glass fibre discs, and the counts then determined by Cerenkov counting. I-RNase assays were carried out using various amounts (10–250 fmol) of ΔKP I-RNA, assayed over various time courses (0–90 min). This enabled the initial rates of degradation of I-RNA to be determined at each substrate concentration. A Lineweaver–Burke plot was then constructed and the K_m for I-RNA determined. Equivalent assays were also performed in the presence of 12 pmol of ΔSL or ΔKP G-RNA competitor. This enabled determination of the apparent Michaelis constant K_{app}, for I-RNA in the presence of the G-RNA competitor, thus allowing the K_i for the competitor (G-RNA) to be calculated using the equation $K_{app} = K_m(1 + [I]/K_i)$, where [I] is the concentration of G-RNA inhibitor.

Composition analysis of I-RNA transcripts

[^{32}P]UTP- or [^{32}P]CTP-labelled RNA transcripts were digested with an excess of I-RNase for 2 h, and the digestion products were fractionated by TLC on PEI plates using 0.65 M sodium formate, pH 3.4 as the solvent (Scadden and Smith, 1995).

Deamination reaction

Equimolar amounts of pBluescript T3 *Asp*718 and T7 *Xba*I RNAs were annealed as described above in 'Constructs and transcripts'. The resultant dsRNA, if correctly annealed, would contain an 82 bp duplex with 5′ overhangs of 39 and 11 nucleotides on the T3 and T7 sides respectively. dsRNA (200 fmol) was deaminated in a 25 μl reaction containing the following components: 0.5 μg of poly(A), 5 mM EDTA, 0.2 μg of bovine serum albumin, 25 mM Tris–HCl, pH 7.9, 100 mM KCl, 5% glycerol, and 6.25 μl of His-tagged recombinant RED-1 (a gift from Mary O'Connell, University of Basel). The reaction was incubated at 30°C for 1 h. The RNA was then extracted with phenol/chloroform, recovered by ethanol precipitation and resuspended in 10 μl of double-distilled water. The dsRNA was denatured prior to subsequent degradation assays by heating at 90°C for 5 min followed by snap cooling on ice. For analysis of the efficiency of the deamination reactions, the RNA was digested with RNase P1 for 1 h at 50°C, and the digestion products fractionated by TLC on PEI plates. The solvent used comprised saturated $(NH_4)_2SO_4$, 0.1 M Na acetate, pH 6.0, and isopropanol (mixed in the ratio 79:19:2). The identity of the spots corresponding to 5′-AMP and 5′-IMP was verified by the migration of unlabelled standards.

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