RNase YI* and RNA structure studies

Vincent J. Cannistraro and David Kennell*

Department of Molecular Microbiology, Washington University School of Medicine, St Louis, MO 63110, USA

Received November 22, 1996; Revised and Accepted February 19, 1997

ABSTRACT

The enzymology of RNase YI*, a recently discovered endoribonuclease from yeast, was studied and compared to other endonucleases for detection of single-strand regions and base pair mismatches in RNA. Its value for RNA structure analyses was assessed with Escherichia coli 5S rRNA as a model substrate. The generally accepted structure of the 5S rRNA is based on thermodynamic energy considerations as well as structures conserved in regions of the molecule during evolution. S1 and mung bean nucleases gave similar results with very marked preference only for the longest single-stranded region in the model. RNase YI* was much more discriminating for detecting unpaired nucleotides as well as short single-strand regions and predicted the generally accepted 5S rRNA structure. Preliminary experiments also indicated that RNase YI* was more sensitive than RNase I for detecting single or multiple base pair mismatches in an RNA–DNA hybrid.

INTRODUCTION

The function of many RNA molecules in biological reactions is defined by their structure as well as their sequence. Secondary structures, such as stem–loops, bulges, mismatches and single-stranded regions can define a function, as can the tertiary conformations of pseudoknots, triple-stranded complexes and non-Watson–Crick base pairings (1–8). Most tertiary structures have been identified using NMR (9) and a few by X-ray crystallography. However, NMR has been limited to small molecules and X-ray analyses have been limited by difficulty obtaining crystals.

The most common probes of RNA structure have been specific chemicals or RNases (10,11). Most of the endoRNases currently available for cleavage of specific phosphodiester bonds do not have the same sequence specificity at all activities, e.g. pancreatic RNase A (12,13), RNase U2 and RNase T2 (14,15) and RNase C13 (16,17). RNase T1 is an exception with its stringent specificity for cleavage after unpaired G. This variable expression of bond preferences can be useful when performed over a wide range of enzyme activities, or alternatively, lead to ambiguous results.

A second class of RNases have no obvious preference for specific phosphodiester bonds but are specific for single or double strands. RNase VI preferentially cleaves RNA double strands (18,19), but it can also degrade single strands in stacked helical structures (20). Many RNases and nucleases are listed as single-strand specific, but for each enzyme it is for a degree of single strandedness. In this paper it is shown that the specificity can range from a slight preference for single strands to an apparent requirement for a long single-stranded region with an intermediate activity that can recognize single base pair mismatches in a duplex.

We describe a yeast enzyme that has such an intermediate recognition. We call the enzyme RNase YI*, because its substrate specificity is similar to that of RNase I* of Escherichia coli. The E.coli enzyme is coded by the rna gene for the periplasmic RNase I (22,23), and it appears to be a cytoplasmic precursor to RNase I which differs not only in cell location but in enzyme specificity and physical characteristics (21). While both E.coli enzymes have no obvious bond preferences, RNase I activity causes significant nicks in double-stranded RNA that lead ultimately to its complete degradation (21). In contrast, RNase I* and RNase YI* are highly specific for small unstructured RNA oligonucleotides. Biochemical analyses have shown the E.coli enzyme functions in vivo in the degradation of the small oligonucleotides (24) derived from mRNA endonucleolytic cleavages (25). Unlike many of the enzymes that have been used for probing RNA structure (10,11,26), RNase I* and RNase YI* do not require cations for activity, are weakly inhibited by NaCl, have pH optima close to neutrality and generate 5’-OH groups upon cleavage for easy end-labeling. In this paper we show that the high selectivity of RNase YI* for unpaired nucleotides makes it a useful probe of RNA structure, and its enzymology is studied to facilitate its use.

MATERIALS AND METHODS

Enzymes and reaction conditions

RNase YI* was purified from Saccaromyces cerevisiae, strain Py26 (Mata, ura3-52, trp1Δ, leu2-3, 112, prb1-1122, ppc1-407, pep4-3, Δnuc1::LEU2). Four liter cultures were grown at 30°C in 1% yeast extract, 2% peptone, 2% glucose to give 20 g wet weight of cells, harvested and extracts prepared in a BioSpec bead beater, as described (27). Subsequent steps were at 0 to 2°C. After removal of most nucleic acids by Polymin P precipitation, the supernatant was brought to 45% ammonium sulfate (27) and the precipitate, containing about half the total protein, was discarded after centrifugation. The supernatant was brought to 90% ammonium sulfate; the resulting precipitate (∼2% peptone, 2% glucose to give 20 g wet weight of cells, harvest, and extracts prepared in a BioSpec bead beater, as described (27). The supernatant was brought to 90% ammonium sulfate; the resulting precipitate (∼2% peptone, 2% glucose to give 20 g wet weight of cells, harvested and extracts prepared in a BioSpec bead beater, as described (27). The supernatant was brought to 90% ammonium sulfate; the resulting precipitate (∼2% peptone, 2% glucose to give 20 g wet weight of cells, harvested and extracts prepared in a BioSpec bead beater, as described (27). The supernatant was brought to 90% ammonium sulfate; the resulting precipitate (∼2% peptone, 2% glucose to give 20 g wet weight of cells, harvested and extracts prepared in a BioSpec bead beater, as described (27). The supernatant was brought to 90% ammonium sulfate; the resulting precipitate (∼2% peptone, 2% glucose to give 20 g wet weight of cells, harvested and extracts prepared in a BioSpec bead beater, as described (27). The supernatant was brought to 90% ammonium sulfate; the resulting precipitate (∼2% peptone, 2% glucose to give 20 g wet weight of cells, harvested and extracts prepared in a BioSpec bead beater, as described (27).
strong binding to the former. Unless noted, RNase YI* was assayed in 10 mM 2-[N-morpholino]ethanesulfonic acid (MES), pH 6.4 (see below). The ammonium sulfate pellet was resuspended in <5 mL of 40 mM Tris–HCl, pH 8.0. If necessary, residual precipitate was removed by centrifugation (here and in subsequent steps) without loss of activity. The clear supernatant was applied to a HiLoad 16/60 Superdex 75 sizing column ($V_t = 122$ mL, $V_0 = 44$ mL) with elution in 20 mM Tris–HCl, pH 7.4 + 200 mM NaCl at 0.5 mL/min. Fractions of 0.7 mL were collected. RNase YI* eluted after a volume (~55 mL) corresponding to $M_r$ ~70 kDa with a purification of ~15-fold. The active fractions (in ~6 mL) were pooled and diluted 4-fold with 20 mM MES, pH 5.7, then loaded, using a 50 mL Superloop immersed in ice, onto a 22 mL S-Sepharose column. After collecting the flow through (~70% of the $A_{220}$) in 15 mM MES, pH 5.7, 2 mL fractions were collected at 0°C at 1 mL/min with a 180 mL linear gradient from 0 to 2 M NaCl in 15 mM MES, pH 5.7. RNase YI* eluted in a peak at ~0.7 M NaCl with a 20-fold purification to yield a total purification of ~4000 U (defined below) (~3 U/µg protein). It lost activity with a half-life of ~8 months in 50% glycerol at ~20°C. Further purification was achieved by dialyzing the active fractions (~8 mL) against 20 mM Tris–HCl, pH 7.4 + 200 mM NaCl and then applying it to a 1 mL monoQ column immersed in ice. The enzyme eluted in 1.5 mL at 0.18 M NaCl in a 40 mL linear gradient from 0 to 1 M NaCl in 20 mM Tris–HCl, pH 7.4 to give another 15-fold purification (~50 U/µg). After >7000-fold total purification, this preparation was probably ~30% pure RNase YI* based on the $A_{220}$ of the activity peak from the monoQ column relative to the intensity of a Coomassie stained band that migrated at ~75 kDa, after SDS–PAGE, but it has not been proven to be the RNase YI* band. If it is, its size agrees with the size estimated by gel filtration and suggests that RNase YI* is a monomeric protein that is large relative to many other endoribonucleases. Almost all the other protein in the SDS gel was in a single band at ~65 kDa. The latter protein eluted from the monoQ at 0.28 M NaCl and accounted for ~40% of the $A_{220}$ applied to the column. The enzyme was dialyzed against 30 mM Tris–HCl, pH 7.4, 40 mM NaCl, 50% glycerol (~4-fold reduction in volume) and stored at ~20°C after adding bovine serum albumin to 100 µg/mL.

The final preparation was free of detectable DNase, phosphatase or RNA exonuclease activities. Single-strand DNase activity was assayed by incubating 10 µg oligo(dC)$_{33}$ with 1 U enzyme in a 20 µL reaction containing 20 mM Tris–HCl, pH 7.4 and 10 mM MgCl$_2$ at 37°C for 2 h before loading on a monoQ column and eluting in a gradient from 0 to 2 M NaCl in 20 mM Tris–HCl, pH 7.4 (21.23). All $A_{220}$ was centered in a sharp peak that eluted at ~0.6 M NaCl at which intact oligo(dC)$_{33}$ elutes. Double-stranded DNase activity assay and reaction conditions were the same using lambda HindIII DNA fragments as substrates. Phosphatase activity was assayed by incubating 1 U enzyme with [γ-$^{32}$P]ATP in 20 mM Tris–HCl, pH 7.4 at 37°C for 2 h and separating any released $^{32}$P from [γ-$^{32}$P]ATP by ascending chromatography at 22°C on a cel 300 PEI sheet (Machery-Nagel) with a solvent of 2 M sodium formate, pH 3.5 (25). There was no evidence of a processive RNA exonuclease activity based on the size distributions of RNA homopolymer products, separated on a monoQ column (29).

RNase I was purified from E. coli strain K-12, containing a plasmid with the rna gene (22.23), and was assayed in 20 mM Tris–HCl, pH 7.4. One unit of RNase I or RNase YI* degrades 1 µg single-stranded homopolymer RNA (~300 nt) to <6mers per min at 37°C in a 20 µL reaction. This definition was equivalent to ~2 U based on a definition of conversion to products soluble in 5% trichloroacetic acid. Reactions with SI nuclease or mung bean nuclease (Boehringer Mannheim) were in 30 mM sodium acetate buffer, pH 5.0, with 1 mM ZnSO$_4$.

**Assay for dinucleotide degradation**

Two kinds of assays were used. The first used 5′-$^{32}$P-XPy-OH-3′ as substrate. RNase YI* generates 5′-$^{32}$P-XP plus 5′-OH-Y-OH reaction products which were separated on cel 300 PEI sheets (Machery-Nagel) by ascending chromatography at 22°C with a solvent of 2 M sodium formate, pH 3.5. The sheets had been pre-washed in 2 M formic acid brought to pH 2.2 with pyridine followed by a final rinsing with water (30). The $^{32}$P-X-OH migrated slower than did the original 5′-$^{32}$P-dimer. The PEI sheets were dried and the positions of labeled products identified by autoradiography, then cut out and counted.

The second assay used unlabeled substrate (5′-OH-XPy-OH-3′). Products of the reaction are 5′-OH-Xp and 5′-OH-Y-OH and were fractionated in a salt gradient on a monoQ column. The 5′-OH-Xp mononucleotide eluted at ~0.14 M NaCl and the dialcohol dimer with only an internal phosphate at 40 mM NaCl. The 5′-OH-Y-3′-OH nucleoside product was not bound.

**Assay for release of 5′-nucleotide from a random mix of RNA oligonucleotides**

Total yeast RNA was partially digested with 0.5 N NaOH for times varying from 0.5 to 15 min at 37°C to produce a uniform distribution of sizes with 5′-OH ends and then 5′-$^{32}$P-labeled with [γ-$^{32}$P]ATP in the polynucleotide kinase reaction. The 5′-$^{32}$P-oligonucleotides were separated by 20% PAGE and an oligonucleotide band of ~25 nt length was eluted to use as substrate. To obtain data for kinetic analysis each reaction contained the same amount of 5′-$^{32}$P plus a known amount of unlabeled yeast RNA carrier which defined the relative concentration of RNA. Reactions were in 20 µl with 14 U RNase YI* for 6 min at 23°C and stopped by adding 1 M citric acid, pH 3.5, to 25 mM and ZnCl$_2$ to 1 mM. Rates were linear with all concentrations during this time. The mononucleotides were separated by PAGE in a pH 3.5, 10% citrate gel, cut out and counted. AMP and CMP did not separate sufficiently and were counted together.

**Structural analysis of E. coli 5S rRNA**

*Escherichia coli* 5S rRNA (Boehringer Mannheim) was treated with heat labile alkaline phosphatase before phenol extraction and ethanol precipitation and then 5′ end-labeled with [γ-$^{32}$P]ATP. The RNA was brought to 100°C for 2 min to dissociate any molecules with ‘nicks’ before loading on a 20% polyacrylamide gel. After electrophoresis, the gel band containing the full-length molecule, was excised, crushed and the RNA eluted by overnight incubation in a small volume of water. The acrylamide particles were spun out and the supernatant precipitated with ethanol and the RNA resuspended in water.

RNase Y* reactions were in 20 µl of 10 mM MES, pH 6.4, at 23°C for 30 min with 1 to 2 µg 5′-$^{32}$P-RNA and stopped by addition of 1 M citric acid to give 30 mM, pH 3.5. After adding 10 µg poly(A), they were brought to 100°C for 1 min and then chilled to 0°C before adding 8 µl of 10 M urea plus 0.2 µl of 0.1 M ZnSO$_4$. The tracking dyes, bromophenol blue and xylene cyanol
and glycerol (to 10%) were added and the samples loaded and run by 20% PAGE at 23°C.

Mismatches in duplex nucleic acids

Plasmid pSP73 (Promega) was linearized by BamHI digestion and used as a template to synthesize a runoff 58 nt RNA from the T7 promoter in vitro in the T7 RNA polymerase (Boehringer Mannheim) reaction in 50 µl containing 40 mM Tris–HCl, pH 8.0, 6 mM MgCl₂, 10 mM dithiothreitol, 2 mM spermidine, 2 mM of each of the four nucleoside triphosphates, 1.25 µg linearized plasmid and 50 U enzyme for 60 min at 37°C. A separate reaction in 20 µl contained the same concentrations of reactants plus 20 µCi [γ-32P]ATP in the polynucleotide kinase reaction. In order to eliminate any possible contaminating 32P fragments, the 32P-RNA was identified in the lanes with 32P and the unlabeled bands at the same position in adjacent lanes were eluted, as described in the preceding section. The T7 RNA was then treated at pH 7.5 with heat sensitive calf alkaline phosphatase (Boehringer Mannheim) for 5 min at 37°C. The alkaline phosphatase was inactivated by heating at 100°C for two min. The RNA was 5′ labeled with 50 µCi [γ-32P]ATP in the polynucleotide kinase reaction. In order to eliminate any possible contaminating 32P fragments, the 32P-RNA was purified by another 20% PAGE and prepared by the preceding steps, except that 10 µg carrier poly(A) was added before ethanol precipitating, and the final sample (~1 µg with ~10⁶ c.p.m.) was used to form an RNA–DNA hybrid with a specific 39mer DNA oligonucleotide that contained one or more base mismatches to the first 39 nt at the 5′ end of the RNA. The DNA oligonucleotides had been synthesized in the Medical School’s Protein Chemistry Laboratory. The RNA–DNA hybrids were purified by electrophoresis through a 20% native polyacrylamide gel. Reactions were at 23°C for 60 min (37°C for 12 min gave the same results). RNase I reactions were stopped with SDS to 0.1% and RNase YI* reactions with final concentrations of 3 M urea, 1 mM ZnCl₂ and 30 mM citric acid, pH 3.5. In both cases, the stop mixtures included glycerol and dyes plus a 10-fold excess of DNA oligonucleotide that had the same sequence as the 39 nt of 32P-RNA. Subsequently, each mix was brought to 100°C for 90 s to dissociate the RNA–DNA hybrid and then to 37°C for 5 min to convert all the released DNA to a DNA–DNA duplex. The 32P-RNA ran as single strands in the subsequent PAGE containing 7 M urea plus 2.5 mM EDTA.

RESULTS

Effectors of RNase YI* activity

pH optimum. The reaction rate of RNase YI* was dependent on pH over a fairly narrow range from 6.0 to 8.0 with a peak of activity at pH 6.2 (Fig. 1). However, since the activity decreased precipitously at pH <6.0, we have used MES, pH 6.4 buffer in most assays. The marked decline in reaction rate at pH 6.0 did not result from inactivation but rather from a slower reaction. The reaction rate was the same in 10 and 50 mM MES and with phosphate or Tris buffers in the pH range that overlapped.

Inhibitors of RNase YI*. The activity was inhibited by various divalent cations, such as 10 mM Mg²⁺, 5 mM Mn²⁺ (~50%) or 0.1 mM Zn²⁺ (>95%). Conversely, it was fully active with 1 mM EDTA, although 10 mM EDTA inhibited 30%. It retained almost full activity in the presence of 200 mM NaCl or KCl salts.

Heat inactivation. RNase YI* is very heat labile. The half life at 37°C in the absence of substrate was ~8 min and at 60°C ~24 s (Fig. 1) but might be longer in the presence of substrate, as is the case with RNase II (29), and it was much more stable to heat in 50% glycerol. This sensitivity to heat could obviate the need for phenol extractions to eliminate enzyme activity.

Substrate specificities of RNase YI*

Homopolymers. RNA homopolymers of A, C or U were degraded by RNase YI* at the same rates (21).

Dimers. The reaction velocities of many broad-specificity endo- or exo-ribonucleases are slower with smaller substrates and may not even react with substrates below some minimal size, e.g. the processive exoRNase II reaction slows markedly when the RNA substrate is less than ~12 nt and the limit digest yields a dimer (29). The 16 possible RNA dimers were tested as substrates for RNase YI* by two different assays which also differed by the presence of 5′-P in one and not the other (Fig. 2). In both cases, RNase YI* was inactive with all dimers except those that had a 5′ G residue (5′-G-X) with no obvious preferences among the four 3′-nucleotides as shown by Lineweaver–Burk plots.

5′-End bond preferences in total cell RNA. The observed bond preferences seen with dimers could carry over to give similar preferences for the 5′-end bonds of longer oligonucleotides. A random mix of oligonucleotides of specific size (~25 nt) were 5′-32P labeled. Equivalent amounts of 32P were incorporated into each of the four 5′-nucleotides as shown by a complete digest with RNase I, or alkali. 5′-32P-A and 5′-32P-C were not resolved, but the sum of their counts was about twice that of 5′-32P-G or 5′-32P-U. However, at low substrate concentrations RNase YI* generated 5′-32P-U four to five times slower than each of the other 5′-nucleotides. The reaction rates for their appearance became closer as the substrate concentrations increased to give the same V_max values and a higher K_m value for cleavage of 5′-U (Fig. 3). These results show that 5′-U residues are cleaved more slowly by RNase YI* than are 5′-A, 5′-C or 5′-G. However, the striking

Figure 1. The pH dependence of RNase YI* in 50 mM MES, 20 mM Tris–HCl or phosphate buffers and heat inactivation of RNase YI*. Activity was estimated from the extent of degradation after fractionation on a monoQ column. (A) The maximum activity is at pH ~6.2 and falls off rapidly at lower pH. (□, MES; (●), phosphate; (○), Tris–HCl). The reactions contained 20 µg polyc(C) and 10 U enzyme in 100 µl at 23°C. (B) Ten units of RNase YI* in 100 µl of 10 mM MES, pH 6.4, was incubated for the time and at the temperatures shown. Samples were brought to 0°C before adding 20 µg polyc(C) and incubating for an additional 10 min at 37°C.
Nucleic acid inhibitors of RNase YI*

Single- or double-stranded DNA were potent inhibitors. As little as 0.4 μg oligo dC25 (s.s.) inhibited >90% of the degradation of 20 μg poly(C) and 1 μg Hind III DNA fragments (d.s.) also inhibited RNase YI* to the same degree. Highly structured RNA was degraded very slowly by RNase YI* (or RNase I*) (21). This specificity could reflect a low affinity to structured RNA, or conversely, the enzyme might bind well but be unable to hydrolyze bonds. In the latter case, structured RNA could inhibit degradation of homopolymer substrates. In experiments with limiting RNase YI*, tRNA was a potent inhibitor; 0.8 μg inhibited by >90% in the same poly(C) assay. However, very high concentrations of poly(C) (300 μg in a 100 ᵎ reaction) were degraded at the same rate with or without 0.8 μg tRNA. The results suggest that tRNA is a competitive inhibitor that binds to the enzyme without being converted to product. It is likely that rapid release of the enzyme requires a hydrolytic reaction. Since duplex RNA or single- or double-stranded DNA are not substrates for cleavage, enzyme remains associated much longer to make them competitive inhibitors at concentrations much lower than that of the substrate.

RNase YI* as a probe of RNA structure

The 120 nt 5S rRNA of *E. coli* has been a model for numerous structure studies since its sequence was first determined (30,31). The generally accepted structure, based on estimated thermodynamic stability and evolutionary conservation of specific segments (32), is shown in Figure 4. The following observations suggest that RNase YI* reactions predict this structure.

Cleavages by RNase YI* from the 5' end. (The numbers refer to nucleotides from the 5' end.) A very intense pair of bands at G-9 and G-10 appeared at the lowest enzyme activities and not only persisted but increased at higher activities (Fig. 5A). These bands must result from cleavages at the end of 'stem I'. NMR analysis of isolated stem I has indicated that G-9, paired with U-111, is not only stacked with G-10 but also with G-112 on the opposite strand rather than to its adjacent C-8 (33). This could distort the phosphodiester bonds of G-9 and U-111 as well as G-10 and C-110 to account for lability at G-9.

There were no bands seen between C-3 and G-9 even at the highest RNase YI* activity indicating that stem I is extremely stable except for 'end-nibbling' to give some dimers and trimers. The dimer 5'-UG-3' accumulated at the highest enzyme activity which is consistent with the preceding observation that 5'-UG-3' is not a substrate. In contrast, there were a series of strong bands from G-9 to A-15 that increased with activity and then declined at the highest activities. These bands resulted from molecules that were cleaved before any cleavages more proximal to the 5' end and were then lost as subsequent cuts trimmed the molecule to the 'stop' at G-9 and G-10. The next pairs of persistent bands were at G-23 and G-24 and at C-26 and C-27.

The most prominent larger molecules appeared as a smear of bands that corresponded to cleavages in the large single-stranded region from A-34 to U-48. They appeared at low enzyme activity and only declined at the highest activity when cleavages occurred in the same molecules more proximal to the 5'-32P end. It was not determined if all the bonds in this region were hydrolyzed with equal rates. However, bands resulting from cleavages near the ends
Figure 4. Structure of 5S rRNA based on energy considerations and the conservation of secondary structures in a broad range of species. The nucleotides are numbered starting at the 5′ end. G to U bonds are designated by a (C). The cleavage sites are determined from Figure 5. Those from the 5′-32P end are designated by pointed arrows; closed arrows show ends that persisted at the highest reaction conditions, and open arrows for bands that declined with increasing enzyme activities. Cleavage sites from 3′-32P ends are shown by the ball arrows with dashed tails showing bands that declined with increasing activities. The arc in the large loop region designates 32P bands that have not been resolved but must include cuts at all bonds. The darker arrows after G-9, G-10 and U-111 designate the most prominent bands which were also the most persistent with increasing activities. The calculated total net free energy is –35.5 kcal/mol (kindly computed by Michael Zuker).

of the loop, i.e. near A-34 and U-48, were stronger which could reflect a rapid loss of single-strand ‘tails’ from internal loop cuts.

Cleavages from the 3′ end. When the 5S RNA was labeled at the 3′ end (Fig. 5B), the most prominent bands were in the single-stranded region from G-112 to G-107. In this case the smallest product seen at the highest activity was the trimer, 5′-A-U-32P-C-OH. Since no evidence of dimer or monomer was visible, this oligo might not be degradable by RNase Y1*. A single declining band appeared at G-102 which is consistent with the single strand that terminates at the stem starting at that base. Finally, there was a very strong band just below the full-length size. It was too high in the gel to calculate an exact nucleotide site, but it results from cleavages in the large loop at A-34 to U-48.

An obvious difference between the 3′ end versus 5′ end labeling was that many fewer cleavages were observed on the 3′ half of the molecule. This difference could result from tertiary interactions between that side of the molecule (U-48 to C-97) that make it less accessible to RNase Y1* activity.

Probing 5S rRNA structure with S1 and mung bean nuclease. The results with S1 and mung bean nuclease were in agreement with each other but were markedly different from the results with RNase Y1* (Fig. 6). Briefly, they showed only one major cleavage region at all activities used: the large loop at C-34 to U-48. There was a secondary 3′-32P-band mapping at G-96 which would result from cleavage between two consecutive G-U bonds. Both primary and secondary bands declined with increasing enzyme activities, but there were no major stops even at G-9 or G-112. Apparently, these enzymes are specific for extended single-stranded regions but are not sensitive to short disruptions of a duplex with the exception of the cleavage at G-96.

The detection of base pair mismatches by RNases

The apparently successful use of RNase Y1* as a probe of RNA secondary structure suggested that it has a strong preference for bonds of unpaired nucleotides. This conclusion was tested with RNA–DNA duplexes with known base pair mismatches. A defined sequence of RNA was synthesized in vitro from the T7 promoter of plasmid pSP73 and hybrid RNA–DNA complexes formed with DNA oligonucleotides containing specific base mismatches. The DNA–RNA hybrids with 32P at the 5′ end of the RNA were reacted with a range of enzyme activities under fairly relaxed hybrid conditions (37°C and no added salt). RNase I has been suggested for detecting base pair mismatches (e.g. by Promega Corp., Ambion, Inc.). It was tested in parallel with RNase Y1*, for identifying either four contiguous mismatched bases or a single mismatched base pair, by the appearance of a specific band in a polyacrylamide gel. Even with a 4 nt ‘bubble’, RNase I degraded it barely faster than it broke phosphodiester bonds of nucleotides in perfect base pairs. The specific band was only observed in a very narrow range of enzyme activities (Fig. 7A). In contrast, RNase Y1* easily identified the four base pair mismatch over a wide range of activities. The single base pair mismatch was also identified by RNase Y1* but was completely missed by RNase I with the activities tested (Fig. 7B).

DISCUSSION

Endonucleases have marked variability for single versus double strands

An ideal enzyme, or combination of enzymes, for elucidating RNA structure should (i) show high preference for any unpaired
nucleotides, (ii) recognize the same phosphodiester bonds at all enzyme activities, and (iii) be active in reaction conditions that do not disrupt the normal RNA structure. As noted, most of the endoRNases used for probing structure have bond preferences that can be a function of the enzyme activity. Of the enzymes that have no obvious bond preferences, the periplasmic RNase I of Escherichia coli has a preference for single strands, but that preference is not sufficiently high, so that the levels of double strand nicks are unacceptable. A single base pair mismatch was not detected and disappearance appeared to be quite ordered, i.e. only specific sites that were vulnerable in the full-length substrate were seen between 3 and 9 nt or 15 and 23 nt. This suggests that the enzyme unsuitable for structure analysis or base pair mismatch detection. At the other extreme, S1 and mung bean nucleases have a very stringent preference for single strands; only bonds in the presumptive long single-stranded loop of the 5S rRNA were hydrolyzed. Short disruptions of double strands were not detected (Fig. 6). Enzymes with such stringent specificity are ideal for eliminating long single-stranded regions without disturbing imperfect duplexes (34) but they are not good candidates for detecting small loops, ‘bubbles’ or single mismatches, and as such, are not good probes for these unpaired nucleotides.

RNase YI* specificity appears intermediate between the extremes of RNase I and these nucleases for single versus double stranded RNA preference. Escherichia coli RNase I* is also in this category, but its value as a probe of RNA structure has not been studied sufficiently. RNase YI* showed little activity against stable duplexes but was able to recognize and cleave short single-stranded regions of the 5S rRNA and recognized the base pair mismatches tested quite well, i.e. it degraded any phosphodiester bonds of nucleotides that were not perfectly bonded in a duplex. This sensitive discrimination for imperfect versus perfect duplexes was also shown by the stability of the latter structures even after multiple cleavages of the 5S rRNA. When a substrate is in great excess, all cleavages should be initial ones. At sufficiently high enzyme activities, some molecules are cleaved more than once, and larger 32P-molecules decline as a result of sufficient enzyme activities, i.e. some cleavages were occurring at G-9 or G-10 in molecules that may have already had several more distal cleavages. This conclusion is apparent because RNase YI* is less stable than perfect duplexes but was able to recognize and cleave short single-stranded regions of the 5S rRNA and recognized the base pair mismatches tested quite well, i.e. it degraded any phosphodiester bonds of nucleotides that were not perfectly bonded in a duplex. This sensitive discrimination for imperfect versus perfect duplexes was also shown by the stability of the latter structures even after multiple cleavages of the 5S rRNA. When a substrate is in great excess, all cleavages should be initial ones. At sufficiently high enzyme activities, some molecules are cleaved more than once, and larger 32P-molecules decline as a result of sufficient enzyme activities, i.e. some cleavages were occurring at G-9 or G-10 in molecules that may have already had several more distal cleavages. This conclusion is apparent because RNase YI* is less stable.
mismatches. It has the added advantages of optimal activity near neutral pH, no requirement for cations that might affect RNA structures, activity in the presence of salt, and the generation upon cleavage of 5'-OH ends for easy labeling.

ACKNOWLEDGEMENTS

We thank Peter M. J. Burgers of the Department of Biochemistry and Molecular Biophysics for the original ammonium sulfate supernatant from which RNase YI* was purified and the Molecular Biophysics for the original ammonium sulfate supernatant from which RNase YI* was purified and the Boehringer Mannheim Corporation for some of the supplies used in this research.

REFERENCES