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The RNA Moiety of Ribonuclease P Is the Catalytic Subunit of the Enzyme

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Summary

The RNA moieties of ribonuclease P purified from both E. coli (M1 RNA) and B. subtilis (P-RNA) can cleave tRNA precursor molecules in buffers containing either 60 mM Mg^{2+} or 10 mM Mg^{2+} plus 1 mM spermidine. The RNA acts as a true catalyst under these conditions whereas the protein moieties of the enzymes alone show no catalytic activity. However, in buffers containing 5–10 mM Mg^{2+} (in the absence of spermidine) both kinds of subunits are required for enzymatic activity, as shown previously. In the presence of low concentrations of Mg^{2+}, in vitro, the RNA and protein subunits from one species can complement subunits from the other species in reconstitution experiments. When the precursor to E. coli 4.5S RNA is used as a substrate, only the enzyme complexes formed with M1 RNA from E. coli and the protein moieties from either bacterial species are active.

Introduction

Ribonuclease P, the enzyme responsible for the maturation of the 5′ termini of tRNA molecules, has essential protein and RNA subunits both in vivo and in vitro (Altman et al., 1982; Stark et al., 1977; Kole et al., 1980; Gardiner and Pace, 1980; Kline et al., 1981; Akaboshi et al., 1980). (M1 RNA and C5 protein are the terms used for the purified RNA and protein subunits respectively of E. coli RNAase P. P-RNA and P-protein are the terms used for the partially purified RNA and protein subunits respectively of B. subtilis RNAase P.)

Characterization of the enzyme, in vitro, has been carried out in buffers containing low concentrations of magnesium ions, i.e. within a factor of 2 of 5 mM Mg^{2+} (Robertson et al., 1972; Stark, 1977; Kole and Altman, 1981; Gardiner and Pace, 1980). The RNA and protein subunits have no ribonuclease activity when assayed separately under standard conditions (buffers containing 10 mM or less Mg^{2+}), but together they can be reconstituted into an active complex (Stark et al., 1977; Gardiner and Pace, 1980). It was suggested that the RNA subunit in this complex plays a role in substrate recognition through the formation of hydrogen bonded nucleotide pairs with tRNA precursor molecules (Reed et al., 1982). The RNA moiety alone, in RNAase P or any other ribonucleoprotein aggregate, was not believed to be capable of performing the catalytic function presumed to be governed by the complex. Recently, however, Cech and coworkers showed that the precursor rRNA found in T. thermophila carries out self-splicing and circularization reactions (Kruger et al., 1982) in the absence of protein.

In this paper we present evidence that RNA may possess a wider range of catalytic capabilities than previously expected. In buffers containing high concentrations of Mg^{2+} the RNA subunits of RNAase P alone are sufficient to carry out the catalytic cleavage of tRNA precursor molecules. However, the protein subunit is required for cleavage activity in standard buffers and in combination with M1 RNA has the ability to cleave one other type of precursor molecule—namely, the precursor to the small, stable 4.5S RNA of E. coli (Bothwell et al., 1976). RNAase P complexes that are active at low Mg^{2+} concentrations can be formed in vitro with the RNA subunit from E. coli and the protein subunit from B. subtilis and vice versa.

Results

RNAase P Activity in the Presence of Low Concentrations of Mg^{2+}

The requirement for both subunits of RNAase P in buffers containing low concentrations of Mg^{2+} (buffers A and B, see Experimental Procedures) is shown in Figure 1. Figure 1A shows the results of assays using a mixture of the RNA precursors to E. coli $tRNA^{Tyr}$ and 4.5S RNA. (Abbreviations used: pTyr, precursor to E. coli $tRNA^{Tyr}$; p4.5, precursor to E. coli 4.5S RNA; pA_3C, synthetic tRNA precursor consisting of pA_3C-$tRNA_F^{Met}$ [E. coli].) Figure 1B shows the results of assays using the synthetic tRNA precursor pA_3C-$tRNA_F^{Met}$ (E. coli). Note that crude RNAase P and reconstituted, purified RNAase P are effective in cleaving both substrates (Figure 1, lanes 1 and 10). A heterologous complex made of M1 RNA from E. coli and P-protein from B. subtilis also cleaves the natural and synthetic substrates (lane 8) effectively. Complexes formed with B. subtilis subunits (lanes 4 and 5), or with P-RNA and C5 protein, the protein subunit of E. coli RNAase P (lane 7), only cleave pTyr under these conditions. Furthermore, M1 RNA by itself appears to have a low level of activity against pTyr and the synthetic substrate (lane 3). This activity is more evident in buffers with high concentrations of Mg^{2+} as shown below. No activity is evident when P-RNA (lane 6), C5 protein (lane 2) or P-protein (Figure 3, lane 5) is used.

It is evident from the results shown in Figure 1 that only native RNAase P or complexes with M1 RNA and either protein (reconstituted by direct mixing, see Experimental Procedures) can cleave p4.5 Neither RNA subunit alone nor complexes made with P-RNA can carry out this particular reaction. This result implies that a protein subunit is needed for p4.5 cleavage, and that the cleavage specificity

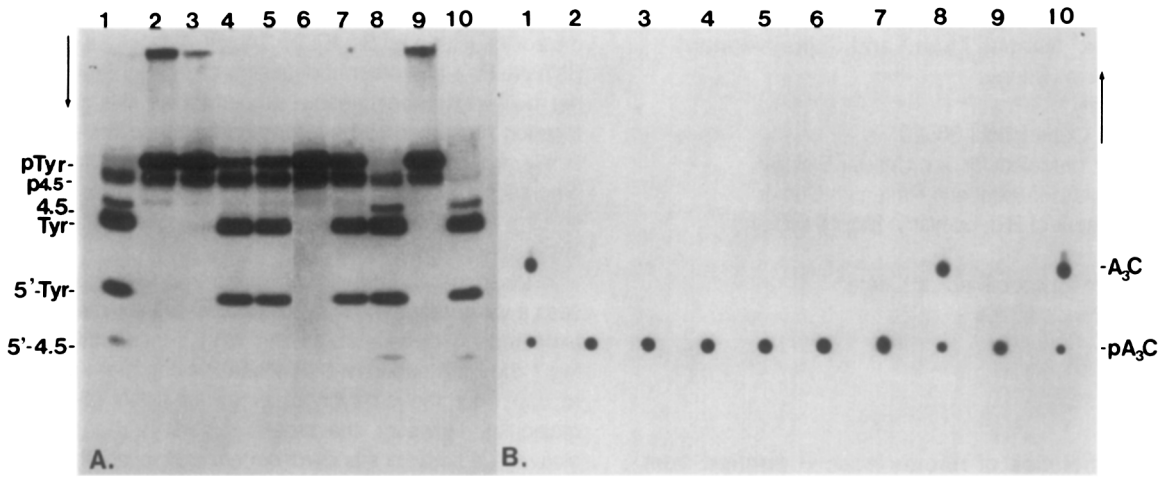

Figure 1. Properties of RNAase P Reactions in 10 mM Mg^{2+} (Buffer B)

Assays for RNAase P activity of complexes reconstituted by direct mixing were carried out and analyzed as described in Experimental Procedures with the additions listed below. (A) Precursors to $tRNA^{Tyr}$ (pTyr) and 4.5S RNA (p4.5) used as substrates. Cleavage products are shown as Tyr and 5′-Tyr; 4.5 and 5′-4.5. Lane 1, E. coli RNAase P (C5 protein + M1 RNA); lane 2, C5 protein; lane 3, M1 RNA; lane 4, B. subtilis RNAase P (P-protein + P-RNA); lane 5, in A same as lane 4, in B, P-protein; lane 6, P-RNA; lane 7, RNAase P (C5 protein + P-RNA); lane 8, RNAase P (M1 RNA + P-protein); lane 9, no addition; lane 10, crude E. coli RNAase P. (B.) pA_3C used as substrate. Cleavage product pA_3C–3′ hydroxyl shown as A_3C. (We note that pA_3C–3′ phosphate, which is nowhere evident in this or following figures has a relative mobility of 0.25 compared to pA_3C–3′ hydroxyl. $tRNA_F^{Met}$, the other product of cleavage, is unlabeled and therefore does not appear in the autoradiogram. Lanes 1–10 as in A, with the exception of lane 5 as indicated above. pA_3C-$tRNA_F^{Met}$ (abbreviated pA_3C), the substrate, is at the origin of chromatography in this and following figures.

RNA \ Protein	E. coli	B. subtilis
E. coli	pTyr +++ p4.5 + pA_3C ++	+++ + ++
B. subtilis	pTyr + p4.5 – pA_3C –	++ – –

direct mixing

RNA \ Protein	E. coli	B. subtilis
E. coli	pTyr +++ p4.5 + pA_3C ++	+++ n. d. —
B. subtilis	pTyr ++ p4.5 – pA_3C –	+++ – –

dialysis

Figure 2. Results of Reconstitution Experiments In Vitro with RNAase P Subunits from Both E. coli and B. subtilis

Reactions were carried out as described in Experimental Procedures. Visual estimates of the relative amounts of RNAase P activity are indicated by plus signs. "RNA" and "protein" refer to the subunits of RNAase P. Left: Reconstitution by direct mixing (assayed in buffer B). Although it is not indicated in this figure. or apparent in Figure 1, most preparations of the homologous B. subtilis complex (P-RNA + P-protein) are indeed capable of cleaving pA_3C in buffer B. Right: Reconstitution by dialysis (assayed in buffer A). Abbreviation used: n.d., not determined.

for tRNA precursors lies entirely with the RNA subunit.

Heterologous RNAase P complexes made by direct mixing, under conditions optimal for reconstitution of B. subtilis RNAase P, or by dialysis, which is optimal for reconstitution of E. coli RNAase P, exhibit enzymatic activity regardless of the sources of the subunits (data summarized in Figure 2). The relative specific activities of these complexes are difficult to calibrate since our B. subtilis subunit preparations are not completely homogeneous. These data are discussed further below.

RNAase P Activity in the Presence of High Concentrations of Mg^{2+}

During a search for the optimal reaction conditions for RNase P we altered the ionic concentration of the reaction buffer in order to enhance the overall secondary and/or tertiary structure of RNA and to provide counter-ion shielding against the expected electrostatic repulsion between the RNAs in the substrate and the enzyme. Test reactions were carried out in buffer containing 60 mM Mg^{2+}, 100 mM NH_4Cl, and 5% glycerol (buffer C, see Experimental Procedures). Remarkably, in this buffer the RNA subunits alone were observed to carry out the cleavage reactions (Figure 3, lanes 3 and 7), although M1 RNA does so more efficiently than P-RNA. P-RNA alone has no detectable activity against the synthetic substrate (Figure 3B). The RNAase P complexes made with M1 RNA and protein from E. coli or B. subtilis both cleave p4.5. This result demonstrated that the RNAase P complexes function as complexes—i.e., both subunits are needed in buffer C in

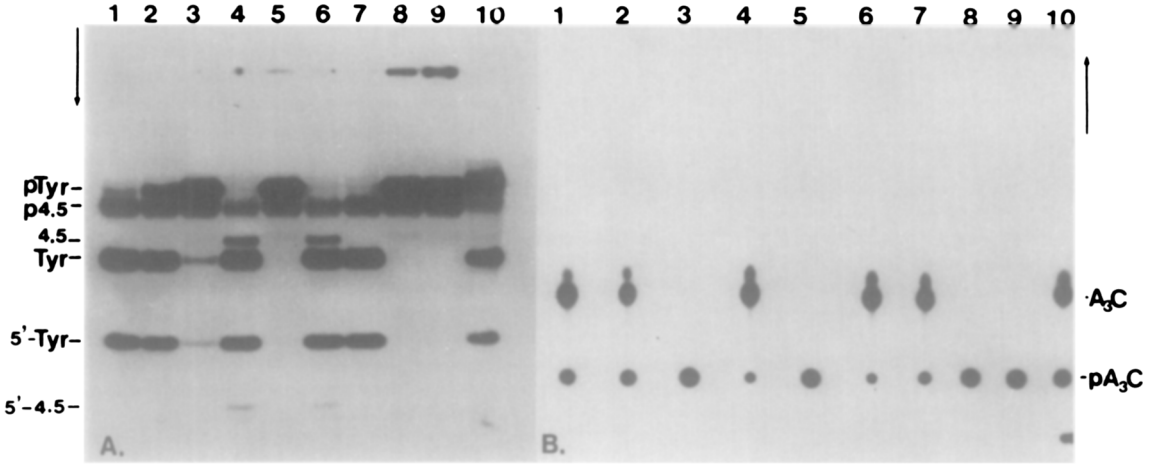

Figure 3. Properties of RNAase P Reactions in 60 mM Mg^{2+} (Buffer C)

Assays for RNAase P activity of reconstitution complexes, made by direct mixing, were analyzed as described in the Experimental Procedures with the listed additions. (A) pTyr and p4.5S were used as substrates. (B) pA_3C used as substrate. Lane legends are the same for each panel. Lane 1, B. subtilis RNAase P (P-RNA + P-protein); lane 2, RNAase P (P-RNA + C5 protein); lane 3, P-RNA; lane 4, RNAase P (M1 RNA + P-protein); lane 5, P-protein; lane 6, E. coli RNAase P (M1 RNA + C5 protein); lane 7, M1 RNA; lane 8, C5 protein; lane 9, no addition; lane 10, crude E. coli RNAase P.

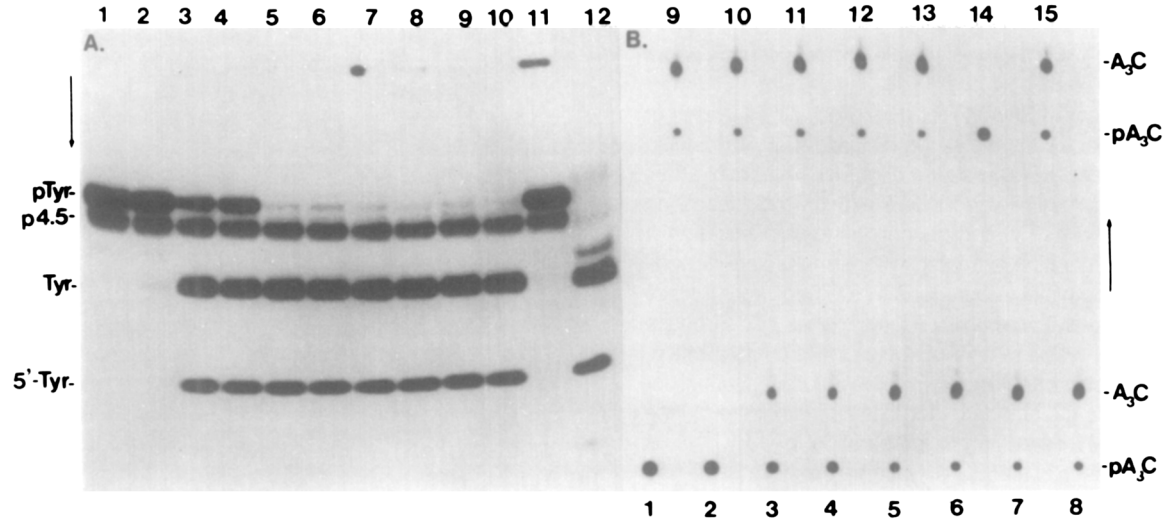

Figure 4. The Mg^{2+} Ion Dependence of the M1 RNA Reaction

M1 RNA (0.18 μg) were incubated for 15 min at 37°C in the presence of pTyr and p4.5 (A) or pA_3C (B) in buffer A (lane 1) or buffer B (all other lanes) containing variable amounts of Mg^{2+} and NH_4Cl as listed: lane 1, 5 mM Mg^{2+}, 100 mM NH_4Cl; lane 2, 10 mM Mg^{2+}, 50 mM NH_4Cl; lane 3, 20 mM Mg^{2+}, 50 mM NH_4Cl; lane 4, 20 mM Mg^{2+}, 100 mM NH_4Cl; lane 5, 30 mM Mg^{2+}, 50 mM NH_4Cl; lane 6, 30 mM Mg^{2+}, 100 mM NH_4Cl; lane 7, 40 mM Mg^{2+}, 50 mM NH_4Cl; lane 8, 40 mM Mg^{2+}, 100 mM NH_4Cl; lane 9, 50 mM Mg^{2+}, 50 mM NH_4Cl; lane 10, 50 mM Mg^{2+}, 100 mM NH_4Cl; lane 11, no additions in A, 60 mM Mg^{2+}, 50 mM NH_4Cl in B; lane 12, crude E. coli RNAase P in A, 60 mM Mg^{2+}, 100 NH_4Cl in B.
(B) Lane 13, 60 mM Mg^{2+}, 100 mM NH_4Cl, 5% glycerol; lane 14, no additions; lane 15, crude E. coli RNAase P.

order to cleave p4.5. Note also that the P-RNA:C5 complex (Figure 3, lane 2) is much more active than P-RNA alone (lane 3). In buffer C, the correlation between the ability to cleave the synthetic and the natural substrates is better than in buffer B. The protein subunits alone manifest no activity but do not inhibit the reaction in buffer C: they are required for activity in buffers A and B. Note also that all the substrates alone show no autocatalytic nuclease activity in any buffer.

Aspects of the Reaction Catalyzed by M1 RNA

The dependence of the cleavage of pTyr by M1 RNA on the concentration of Mg^{2+} is shown in Figure 4. In 5 mM Mg^{2+} (lane 1) there is no apparent activity, but as the concentration of magnesium ions is increased the activity of RNAase P increases until it reaches a maximum at about

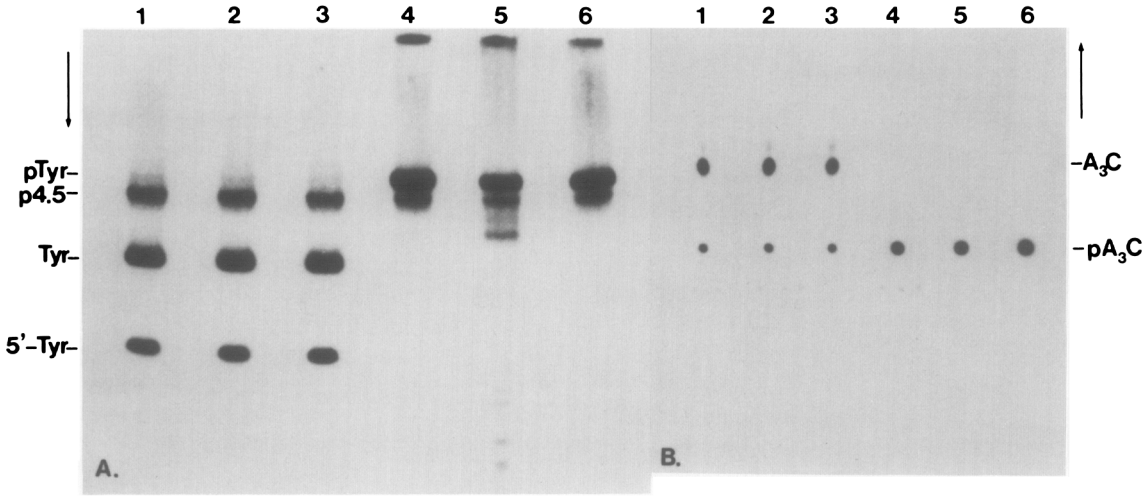

Figure 5. Effect of Protease Treatment on Cleavage by M1 RNA

pTyr and p4.5S used as substrates in the experiments shown in A, and pA_3C used as substrate in the experiments shown in B. Lane legends the same for each panel. Lane 1: M1 RNA (0.28 mg/ml) preincubated with pronase (1 mg/ml) for 30 min at 37°C in TEB buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA). One microliter of this mixture was then assayed in buffer C as described in Experimental Procedures. Lane 2: M1 RNA (0.28 mg/ml) incubated with pronase (0.5 mg/ml) and 1 μl assayed as described above. Lane 3: untreated M1 RNA (0.28 mg/ml) preincubated and assayed as above. Lane 4: bovine serum albumin (0.75 mg/ml) pretreated with pronase (1 mg/ml) for 30 min at 37°C and assayed as above. No M1 RNA added. Lane 5: pTyr incubated with pronase (1 mg/ml) for 30 min at 37°C. Lane 6: pTyr incubated as above with no additions.

50 mM Mg^{2+}. M1 RNA is unable to cleave p4.5 under these conditions.

Pretreatment of RNAase P complexes with RNAases or proteases inactivates the enzyme (Stark et al., 1977; Gardiner and Pace, 1980; Kline et al., 1981; Akaboshi et al., 1980). The M1 RNA used in the experiments described was treated with SDS and phenol during its purification. If the RNA preparation is treated with pronase under conditions that completely degrade BSA (0.75 mg/ml), it loses none of its catalytic capability (Figure 5 lanes 1, 2). Pronase alone, which has a low level of ribonuclease contamination, has no specific effect on pTyr when incubated with it for 30 min, twice the standard incubation time (Figure 5, lane 5), or on M1 RNA. If our preparation of M1 RNA is electrophoresed in an SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue, no protein contaminant is apparent. The limits of resolution of this experiment indicate that less than 1% by weight of the M1 RNA preparation can be protein (data not shown).

The presence of 0.1% SDS in the M1 RNA–pTyr reaction mixture has no effect on the cleavage reaction. Nucleotide triphosphates are inhibitory at 20 μM. U2, an snRNA (Busch et al., 1982) and bulk E. coli tRNA cannot carry out the RNAase P reaction (data not shown). Bulk tRNA inhibits both the RNAase P reaction (Stark, 1977) and the M1-RNA-governed reaction. However, spermidine, when added to buffer B, elevates the rate of cleavage by M1 RNA to that in buffer C. This small polyamine does not confer the ability to cleave p4.5 on M1 RNA at any concentration tested (Figure 6, lanes 5 and 6). Note that spermidine is effective in stimulating cleavage by M1 RNA at molar concentrations much lower than Mg^{2+} ion. S1, a ribosomal protein (Yuan et al., 1979), and BSA do not stimulate cleavage of pTyr by M1 RNA (data not shown).

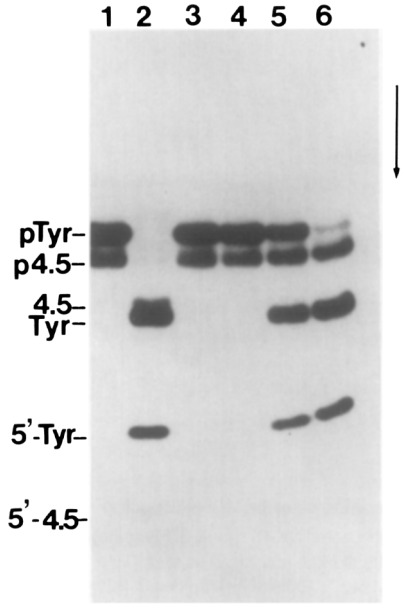

Figure 6. Effect of Spermidine and Mg^{2+} on the M1 RNA Cleavage Reaction

Reactions were carried out in buffer B in the absence of magnesium and with the listed additions and analyzed as described in Experimental Procedures. pTyr and p4.5 were used as substrates. Lane 1, 10 mM Mg^{2+}; lane 2, crude E. coli RNAase P, 10 mM Mg^{2+}; lane 3, M1 RNA, 1 mM spermidine; lane 4, M1 RNA, 5 mM spermidine; lane 5, M1 RNA, 10 mM Mg^{2+}, 1 mM spermidine; lane 6, M1 RNA, 10 mM Mg^{2+}, 5 mM spermidine.

Identity of the Reaction Product

RNAase P processing events in vivo and in vitro release 5′ phosphate and 3′ hydroxyl groups. The same end

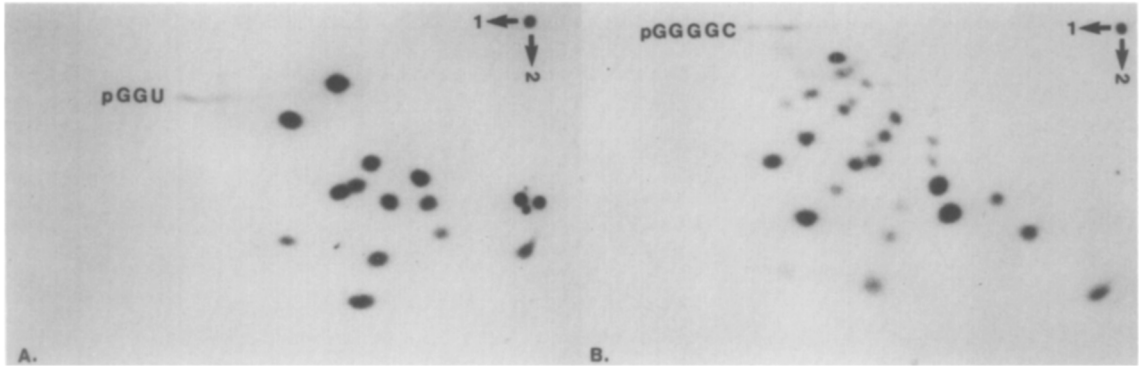

Figure 7. Fingerprints of M1 RNA and RNAase P Reaction Products

Reaction products were treated with RNAase A and chromatographed as described by Platt and Yanofsky (1975). (A) $tRNA^{Tyr}$ product generated by the action of M1 RNA on pTyr. (B) 4.5S RNA product generated by the action of reconstituted E. coli RNAase P on p4.5. The 5′-terminal oligonucleotide is indicated in each case. The p4.5 preparation and the product of the reaction with M1 RNA + C5 protein are contaminated with another host cell RNA species at a low level, which accounts for the appearance of some of the faint spots in the fingerprint. Oligonucleotides unique to 4.5S RNA were identified by secondary analysis with RNAase T_1. First dimension: electrophoresis on cellulose acetate. Second dimension: homochromatography on PEI sheets.

groups are created in vitro in the products of the RNA-governed reactions we describe here. The oligomer pA_3C_{OH} is released from the substrate pA_3C (Figs. 1, 2) by both M1 RNA and P-RNA. The reaction products generated by M1 RNA from pTyr or M1 RNA + C5 protein from p4.5 were separated electrophoretically in polyacrylamide gels, and products, which contained the sequences of mature $tRNA^{Tyr}$ and 4.5S RNA, were fingerprinted by the method of Platt and Yanofsky (1975). The fingerprints contain the expected catalog of oligonucleotides, with none added or missing, and with the expected mobilities (Figure 7). In particular, the 5′ terminus of the product of cleavage of pTyr is pGGU, as determined by secondary analysis of the oligonucleotide marked in Figure 7A, and the 5′ terminus of the p4.5 reaction product is pGGGGC (Griffin, 1975; Bothwell, 1976) as determined in a similar fashion (Figure 7B). (M. Fournier [personal communication] has suggested that this sequence should be pGGGGGC on the basis of results of DNA sequence analysis of the gene for 4.5S RNA.) Aside from the single cleavage of each substrate, it appears that no other chemical change occurred during the M1-RNA-governed reaction. We have shown in separate experiments that M1 RNA itself is also not changed in size during the RNAase P reaction (C. Guerrier-Takada, unpublished).

Kinetics of the Reaction Catalyzed by M1 RNA

We undertook a study of the kinetics of the reaction catalyzed by M1 RNA to determine if the cleavage event had the properties of a true enzymatic reaction and to compare it with the reaction of E. coli RNAase P in buffer A. Assays were quantitated as described in Experimental Procedures by the measurement only of the amount of pTyr and its cleavage product generated in individual reaction mixtures. The reactions catalyzed by E. coli RNAase P and by M1 RNA proceed linearly (Figure 8A), but comparison of optimal conditions for each reaction reveals that the reaction catalyzed by the RNAase P complex has a larger initial velocity. When these two reactions are compared in buffer C, the initial velocities are the same (data not shown). The concentration of "enzyme" used in all the experiments shown in Figure 8 was, at most, less than one sixth of the substrate. If more substrate is added to the M1 RNA reaction 10 min after the start of the initial incubation, cleavage proceeds in a linear fashion beyond what would be expected if no additional substrate had been added to the reaction mixture (Figure 8B). In this experiment at least four times the number, in picomoles, of product was generated compared to the amount of enzyme in the reaction mixture. We have also determined the K_m and V_{max} of the M1 RNA and RNAase P reactions using classical Lineweaver-Burk plots as shown in Figure 8C. The maximum velocity of each enzyme differs, and we have calculated a turnover number of 1 mole product per minute per mole enzyme for M1 RNA and 2 mole product per minute per mole enzyme for RNAase P. The K_m for each reaction has the same value, as shown by the intercepts on the abscissa in Figure 8C (the accuracy of the individual data points is plus or minus 5%). The value for K_m, 5×10^{-7} M, is close to that determined by Stark (1977) for cruder preparations of E. coli RNAase P, indicating that M1 RNA alone may govern the binding of the enzyme to tRNA precursor substrates. Note that the kinetics of the reaction with M1 RNA do show a short lag (Figure 8B) before the reaction proceeds linearly, suggesting that there is a slow initial step in the binding of M1 RNA to its substrate. This lag is not apparent when the RNAase P complex governs the reaction or when M1 RNA has been dialyzed out of 7 M urea before use (Figure 8A). The protein subunit, therefore, probably plays some role in determining the conformation of M1 RNA and its consequent effectiveness in initial binding to substrates in low concentrations of Mg^{2+}.

Discussion

Genetic and complementation analyses in vitro of E. coli mutants that are thermosensitive for RNAase P indicate

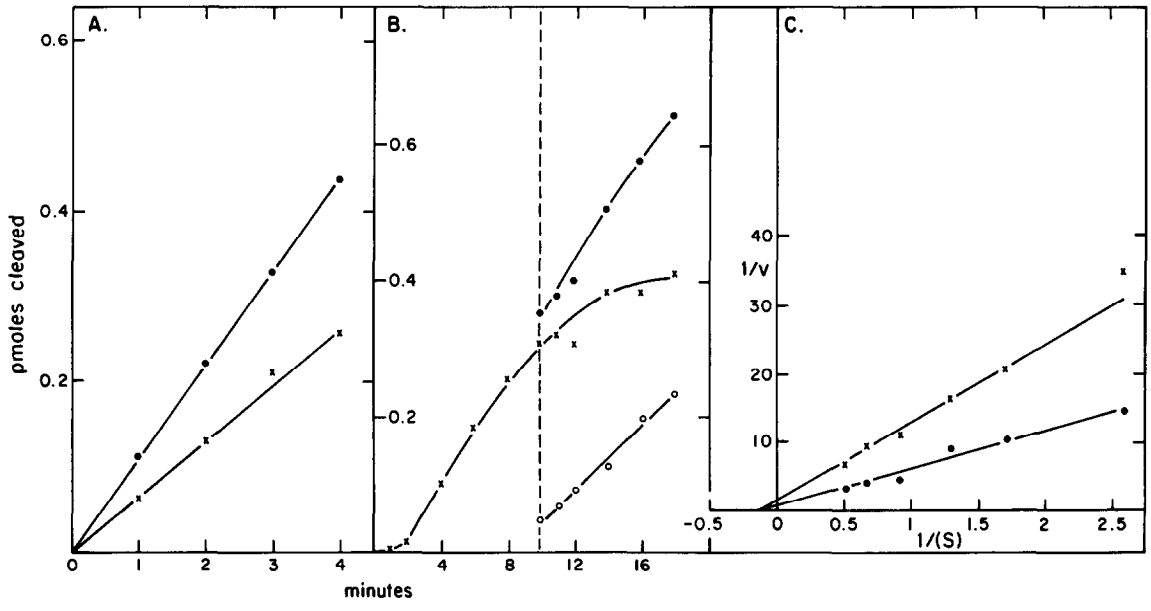

Figure 8. Kinetic Analysis of the M1 RNA and RNAase P Reactions with pTyr Substrate

(A) Comparison of the kinetics of reconstituted (by dialysis) E. coli RNAase P in buffer A (5 mM Mg^{2+}) and M1 RNA in buffer C (60 mM Mg^{2+}). C5 protein (3 pmole) and M1 RNA (3 pmole) were mixed in urea buffer (40 μl final volume) and dialyzed against 4B buffer as described in Experimental Procedures. M1 RNA (3 pmole) alone was treated in a similar fashion. Ten microliters of the reconstitution mixtures (containing 0.75 pmole M1 RNA) was added on ice to 90 μl of the appropriate reaction mixture containing pTyr (3.86 pmole). Twenty microliters was withdrawn, and this was considered the zero time point. The samples were then incubated at 37°C, and 20 μl aliquots were sampled at the indicated times and added to 5 μl of dye solution for electrophoresis. Electrophoresis was carried out in a 10% polyacrylamide gel in 89 mM Tris-borate, 2.5 mM EDTA buffer. After autoradiography, the appropriate bands were excised from the gel and the radioactivity in each determined by counting Cerenkov radiation. (●) RNAase P cleavage of pTyr; (×) M1 RNA cleavage of pTyr.

(B) Kinetics of M1 RNA action in buffer C (60 mM Mg^{2+}). M1 RNA (1.07 pmole) was incubated with pTyr (5.5 pmole) in 200 μl buffer C. Aliquots of 20 μl were withdrawn at the indicated times. Ten minutes after the start of incubation at 37°C further pTyr (2.9 pmole in 40 μl buffer C), or 40 μl buffer C alone, was added to 100 μl of the reaction mixture to make a final volume of 140 μl containing 0.75 pmole M1 RNA and either 6.76 or 3.86 pmole pTyr, and 20 μl aliquots were sampled from each mixture at the times indicated. The samples were analyzed and the products qunatitated as indicated above. (●) pTyr added after 10 min; (×) buffer C alone added after 10 min; (O) net added pTyr cleaved after 10 min (in picomoles).

(C) Determination of K_m and V_{max} for the reactions shown in A. Substrate (pTyr) concentration was varied in reaction mixtures as described in A, and a Lineweaver-Burk double reciprocal plot was constructed from the data. (●) RNAase P in buffer A; (×) M1 RNA in buffer C. Units: 1/S (pmoles × 5 × 10^{-4})$^{-1}$; 1/v [(pmoles substrate cleaved/min)$^{-1}$].

that both a protein and an RNA subunit of the enzyme are essential for RNAase P function and tRNA biosynthesis (Schedl and Primakoff, 1973; Sakano et al., 1974; Ikemura et al., 1975; Kole et al., 1980). However, we have shown that M1 RNA alone can cleave the tRNA precursor in vitro in buffers containing 60 mM Mg^{2+} or 10 mM Mg^{2+} + 1 mM spermidine. Under standard conditions, in vitro, the protein subunit is necessary for the tRNA precursor cleavage reaction, and under all conditions, in vitro, for the cleavage of the precursor to E. coli 4.5S RNA. We infer that reaction conditions in vivo more accurately reflect our standard reaction conditions in vitro, rather than those found in buffer with high Mg^{2+} ion concentration, and that there is no incompatibility between the previously reported genetic data and our new results. It is possible that in addition to conferring some substrate specificity on the complex, in vivo, the protein subunit of RNAase P plays a role analogous to that played in vitro by spermidine.

The successful complementation experiments in vitro with RNAase P subunits from E. coli and B. subtilis demonstrate the importance of the structure in solution of the RNA subunits. Cross-hybridization experiments using DNA containing the cloned M1 RNA gene and B. subtilis genomic DNA are negative (R. Reed, K. Gardiner, N. Pace, and S. Altman, unpublished experiments), indicating that there is no extensive primary sequence homology between the genes for the RNAase P RNA subunits in the two organisms. Their similar functional attributes probably derive from similar structures in solution.

The statement that RNA may be the catalytic subunit of RNAase P is supported by direct experimental evidence. Our data show that RNA which has been SDS-phenol extracted (and, in the case of M1 RNA, also treated with a protease) retains the ability to carry out the canonical RNAase P reaction. The synthetic substrate used is also cleaved by these RNAs but not as efficiently as the natural tRNA precursor. (A similar observation regarding the relative ability of RNAase P to cleave artificial substrates [Kline et al., 1981] had been made previously with intact RNAase P under standard conditions [Kole and Altman, 1980].) Furthermore, the rate of the reaction catalyzed by M1 RNA alone differs only by a factor of 2 from that of the intact RNAase P complex, although M1 RNA has no detectable contaminant protein when examined on SDS-polyacryl-

amide gels. Additionally, our observations that the specificity of reactions catalyzed by RNA are sensitive to the species origin of the RNA (and to the presence of the protein subunit for the reaction with p4.5 as discussed below) support the hypothesis that the reactions are characteristic of the RNA involved and are of biological importance.

We have previously shown that RNAase P activities accurately recognize tRNA precursor substrates from different species (Altman et al., 1982). However, when we use an E. coli–specific non-tRNA precursor substrate, the precursor to 4.5S RNA, P-RNA from B. subtilis is incapable of performing the appropriate cleavage when combined with any protein. M1 RNA does perform the reaction but only when complexed with protein. These results show that the catalytic capability against species-specific substrates resides in the homologous RNA moiety and that a protein is necessary as a cofactor in the reaction. In addition, it appears that mechanisms proposed to explain RNAase P–tRNA precursor recognition can be separated from explanations of the nature of recognition of the precursor for 4.5S RNA, since the nature of the enzyme involved in each case is different.

The results of a kinetic analysis of the reaction governed by M1 RNA show that this RNA behaves as a canonical enzyme. An important aspect of these experiments, however, is the observation that the K_m for the reaction with pTyr is the same as that measured for the intact E. coli RNAase P complex with the same substrate. This result can be interpreted to mean that the RNA alone governs binding to the enzyme.

It is still possible to argue that there are trace quantities of an unidentified protein in our RNA preparations and that this protein is responsible for RNAase P activity. With respect to our results with M1 RNA, however, such a protein would have to be capable of surviving SDS-phenol treatment, digestion with pronase, and would have to be functional in 0.1% SDS. The probability that all these conditions would be met is unlikely, but if they were, one additional experiment is possible that could help to remove doubts concerning the existence of trace quantities of a cryptic protein. M1 RNA transcribed in vitro could be tested as the catalyst in the M1-RNA-governed reaction. The results of preliminary experiments show that the precursor to M1 RNA (Reed and Altman, 1983) is capable of catalyzing the cleavage of pTyr. Further experiments of this nature are in progress.

Both M1 RNA and P-RNA are capable of cleaving more than one substrate. These RNAs differ from the published characteristics of the precursor to T. thermophila rRNA which acts only on itself (Kruger et al., 1982). M1 RNA satisfies the definition of a true enzyme (Fruton and Simmonds, 1958, p. 211) in that it is unchanged (in size) during the course of the reaction; it is needed in only small amounts; it is catalytic, and it is stable. We have no direct evidence as to whether there is transfer or exchange of, for example, phosphate groups between parts of the substrates and/or M1 RNA during the course of the reaction.

Except for a trivial reason, novelty, should we be surprised that an RNA molecule is an enzyme? There are relatively few chemical constraints on the macromolecular nature of catalytic surfaces. Proteins are enzymes; carbohydrates can be altered to be catalytic agents (Breslow, 1982); RNA can have catalytic capabilities. In the primeval replicating system we might suppose that there were crude or nonexistent signals that controlled the initiation and termination of replication and/or transcription (the latter presuming the "genome" was DNA and not RNA). In such a system the generation of functional entities of defined size might well have been left to various nucleic acid cleavage reactions, such as those that exist today in mammalian mitochondria (Ojala et al., 1980). If proteins were relative latecomers in the evolution of macromolecules, then primeval manipulations of nucleic acids may have been carried out entirely or predominantly by catalytic nucleic acids themselves. The remnants of these early, important, biological events may be apparent in the reaction of RNAase P, in the processing of some rRNAs, in ribosomes (though somewhat occluded in this case by the presence of protein), and undoubtedly in other reactions catalyzed by RNA that remain to be identified.

Experimental Procedures

Bacterial Strains

E. coli MRE600 used in the preparation of RNAase P was obtained from the Grain Processing Corp. CT900 (Reed et al., 1982) was used in the preparation of M1 RNA.

Chemicals

Acrylamide and bisacrylamide were purchased from Biorad Corp. Ultrapure urea was obtained from Schwarz-Mann. DEAE-Sephadex and other chromatographic media were purchased from Pharmacia. Radioactive materials were obtained from New England Nuclear or Amersham Radiochemicals Corp. All other chemicals were reagent grade.

Radioactive Substrates for RNAase P

The preparation of the precursor to E. coli $tRNA^{Tyr}$ and estimates of its concentration from specific radioactivity measurements were carried out as described by Robertson et al (1972). The small, stable 4.5S RNA in E. coli has a precursor molecule that is similar in size to pTyr (Bothwell et al., 1976) and is present in pTyr preparations in varying amounts. p4.5S is cleaved in vitro by RNAase P to generate mature 4.5S RNA and accumulates in mutants thermosensitive for RNAase P (Ikemura et al., 1975). When pTyr precursors are electrophoresed at room temperature, the two precursor species are well separated as shown in Figure 1.

Preparation of the Synthetic tRNA Precursor A_3C-$tRNA_F^{Met}$

E. coli $tRNA_F^{Met}$ (10 μg; Sigma) and A_3C_{OH} (90 nmole; kindly provided by Dr. O. C. Uhlenbeck) were incubated 14 hr at 4°C with 50 units of RNA ligase (New England Nuclear) in 500 μl 500 mM HEPES, pH 7.5; 10 mM $MgCl_2$; 3.3 mM DTT; 21 nM ATP. Following phenol extraction and concentration by ethanol precipitation, the product was resolved by polyacrylamide gel electrophoresis and recovered by elution as previously detailed (Stahl et al., 1980). The A_3C-$tRNA_F^{Met}$ (0.5 μg) was labeled at the 5′ terminus with 1 mCi γ-^{32}P-ATP and polynucleotide kinase and isolated by polyacrylamide gel electrophoresis as detailed previously (Stahl et al., 1980).

Preparation of Nonradioactive M1 RNA

M1 RNA was prepared as described by Reed et al. (1982). The concentration of M1 RNA solutions was determined by measuring OD_{260}.

Preparation of C5 Protein

RNAase P purified by DEAE Sephadex column chromatography and concentrated by ammonium sulfate precipitation (Robertson et al., 1972) was used as the source of C5 protein. Total protein (300 μg) was loaded on each preparative polyacrylamide gel (20 × 40 × 0.1 cm). Each gel consisted of a 12.5% separation gel and a 6% stacking gel in 0.1% SDS, 4 M urea generally as described by Laemmli (1970) but with the following modifications. The running buffer contained 0.1% 2-mercaptoethanol; both separation and stacking gels contained 1 mM EDTA and 4 M urea. The protein sample was electrophoresed at 5 V/cm for 15–18 hr until the tracking dye (bromophenol blue) had migrated 30 to 35 cm into the separation gel. Two side lanes, one of which contained myoglobin as a molecular weight marker, were stained with Coomassie Brilliant Blue. The stained band corresponding to C5 protein (Kole and Altman, 1981) was excised from the gel and electroeluted into 1 mM Tris-HCl, pH 7.5; 5 M urea; 0.1% 2-mercaptoethanol. Elution was carried out for 5 hr at 200 V and 4°C. The eluate was dialyzed against three changes of 4 liters H_2O. Aliquots were then analyzed on a 12.5% SDS–urea gel to determine the purity of C5 protein. To eliminate traces of SDS in these preparations, we adapted the procedure of Hager and Burgess (1979). Approximately 1.5 ml of eluted, dialyzed protein were lyophilized and resuspended in 200 μl dilution buffer (50 mM Tris-HCl, pH 7.5; 20% glycerol; 0.15 M NaCl; 1 mM DTT; 0.1 mM EDTA). After addition of carrier BSA (15 μg) the proteins were precipitated with 1 ml cold acetone and the mixture was kept in a dry ice–ethanol bath for 20 min (or overnight at −20°C) and then centrifuged for 5–10 min in an Eppendorf microfuge. The supernatant was discarded, and the pellet dried down and resuspended in urea buffer (50 mM NaOAc, pH 7.2; 7 M urea; 0.5 M NaCl) or buffer B (see below).

Preparation of P-Protein

B. subtilis paste (100–200 g) was suspended in 100–200 ml PA_{200} buffer (50 mM Tris-HCl, pH 7.6; 200 mM NH_4Cl; 10 mM $MgCl_2$; 5% glycerol) at 0°C. Subsequent operations were carried out at 4°C. The suspension was passed once through a French pressure cell (Aminco), adjusted to contain 1 μg/ml DNAase (Sigma, RNAase-free) and clarified by centrifugtion at 39,000 × g. The supernatant volume was adjusted to 450 ml by the addition of PA_{200} buffer, ribosomes were removed by centrifugation at 100,000 × g for 5 hr, and the supernatant (S100) was dialyzed overnight against PA_{60} buffer (50 mM Tris-HCl, pH 7.6; 60 mM NH_4Cl; 10 mM $MgCl_2$; 5% glycerol). The dialyzed cell extract was loaded onto a DEAE-cellulose column (60 cm × 3.2 cm) equilibrated with PA_{60} buffer. The column was washed with 500 ml PA_{60} buffer, then RNAase P activity was eluted with a 4 liter NH_4Cl gradient (200–500 mM) in PA buffer. The RNAase P activity was located by direct assay of 2–5 μl of the fractions, and peak fractions were pooled and concentrated by precipitation with 2 volumes of ethanol. The precipitate was collected by centrifugation, dried briefly under vacuum, and resuspended in PA_{60} buffer to 600 A_{260} units/ml. Protein then was extracted with 66% acetic acid, dialyzed into UP buffer (6 M urea, 50 mM NaH_2PO_4; 10 mM methylamine; 4 mM mercaptoethanol, pH 4.5) and concentrated in an Amicon filtration unit using YM-2 membrane, all as described for ribosomal proteins (Zimmermann, 1979). The protein mixture (10 mg/ml), which was used in the experiments described here, was about 0.5% P-protein, as assayed by densitometric scanning of silver-stained polyacrylamide gels (Oakley et al., 1980), using homogeneous P-protein (T. Marsh, unpublished) as a reference.

Preparation of P-RNA

The S100 supernatant (see above) from 150 g B. subtilis paste was adjusted to 0.02 M in EDTA and extracted three times with phenol, and the RNA was recovered by ethanol precipitation. The precipitate was dissolved in 10 mM Tris-HCl, pH 7.6, 3 mM EDTA, to a concentration of 7 mg/ml, and 35 mg was chromatographed on a Sephadex G-150 column (95 cm × 2.2 cm), equilibrated in 100 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA. Fractions containing the P-RNA were determined by complementation of P-protein in the RNAase P reaction. Active fractions from five such columns were pooled and rerun on the same G-150 column. Active fractions were pooled and concentrated by ethanol precipitation. The P-RNA at this stage of purification (4 mg/ml) was about 5% pure, as estimated by recovery from subsequent preparative polyacrylamide gel electrophoresis. RNA purified through the Sephadex G-150 column was used in all experiments described here.

Assay for RNAase P Activity of Reconstituted Enzyme Complexes

Reconstitution was performed either by direct mixing of subunits or by a dialysis procedure (Kole et al., 1980). Direct mixing: RNA and protein subunits, purified as described above, were incubated in the presence of the appropriate substrate for 15 min at 37°C in 20 μl of buffer B (50 mM Tris-HCl, pH 7.6; 60 mM NH_4Cl; 10 mM Mg^{2+}) or buffer C (50 mM Tris-HCl, pH 7.6; 100 mM NH_4Cl; 60 mM Mg^{2+}, 5% glycerol). RNA subunits alone or protein subunits alone were treated similarly. For assays containing pTyr as substrate, 5 μl of stop dye solution (Robertson et al., 1972) were added prior to electrophoresis in a 10% polyacrylamide gel in 89 mM Tris-borate, 2.5 mM EDTA, run at room temperature. The assays containing pA_3C as substrate were stopped with 100 μl 3 mM EDTA, and aliquots of 15 μl were analyzed by PEI thin layer chromatography as described by Stahl et al. (1980). Dialysis procedure: RNA and protein subunits were mixed or resuspended separately in the urea buffer (40–100 μl) described above and dialyzed for 2 hr against 4B buffer (50 mM Tris-HCl, pH 7.5; 10 mM $Mg(OAc)_2$; 500 mM NH_4Cl; 6 mM 2-mercaptoethanol). Aliquots of 2 μl were then assayed for RNAase P activity in 20 μl of assay mixture that contained either buffer A (30 mM Tris-HCl, pH 7.0; 50 mM NH_4Cl; 5 mM $MgCl_2$; 0.1 mM EDTA; 0.1 mM 2-mercaptoethanol) or buffer C. The assays were analyzed as described above.

Assay for M1-RNA-Directed Cleavage on Substrates

M1 RNA activity was assayed in buffer C in the presence of pTyr (and p4.5) or pA_3C as substrates. Incubations were carried out at 37°C for 15 min unless otherwise indicated and analyzed as described above.

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Note Added in Proof

We have confirmed that the precursor to M1 RNA, prepared by transcription in vitro of the gene for M1 RNA, can cleave pTyr with the same rate as M1 RNA. We have also shown that M1 RNA and the precursor to M1 RNA can cleave at least six tRNA precursor molecules in addition to the ones reported in this paper. These results will be described in full elsewhere.

Unlike the case for the M1 RNA–pTyr reaction (see text), we have shown that 0.1% SDS completely inhibits the cleavage of pTyr by reconstituted RNAase P (M1 RNA plus C5 protein).