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A novel tertiary interaction in M1 RNA, the catalytic subunit of *Escherichia coli* RNase P

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ABSTRACT

Phylogenetic covariation of the nucleotides corresponding to the bases at positions 121 and 236 in *Escherichia coli* RNase P RNA (M1 RNA) has been demonstrated in eubacterial RNase P RNAs. To investigate whether the nucleotides at these positions interact in M1 RNA we introduced base substitutions at either or at both of these positions. Single base substitutions at 121 or at 236 resulted in M1 RNA molecules which did not complement the temperature-sensitive phenotype associated with *rnpA*49 *in vivo* whereas wild-type M1 RNA or the double mutant M1 RNA, with restored base-pairing between 121 and 236, did. In addition, wild-type and the double mutant M1 RNA were efficiently cleaved by Pb^{++} between positions 122 and 123 whereas the rate of this cleavage was significantly reduced for the singly mutated M1 RNA variants. From these data we conclude that the nucleotides at positions 121 and 236 in M1 RNA establish a novel long-range tertiary interaction in M1 RNA. Our results also demonstrated that this interaction is not absolutely required for cleavage *in vitro*, however, a disruption resulted in a reduction in cleavage efficiency (k_{cat}/K_m), both in the absence and presence of C5.

INTRODUCTION

The tRNAs in prokaryotes are transcribed as precursors and various enzymes participate in the processing of these precursors to generate functional tRNA molecules (1). RNase P is the only known enzyme responsible for the maturation of the 5′-termini of all known tRNAs in prokaryotes. The catalytic subunit of RNase P is an RNA molecule, which under certain *in vitro* conditions cleaves various precursors at the correct position in the absence of the protein subunit (2). The protein subunit of RNase P (in *Escherichia coli* C5) is, however, essential for RNase P function *in vivo* (3). The best studied RNase P RNA is the *E.coli* M1 RNA and the function of this ribozyme has been studied in several reports (4, and references therein). To understand its function it is important to elucidate its structure. A secondary structure model based on phylogenetic studies has been constructed. This model includes two tertiary interactions which have been verified by mutational covariations (5, 6, see also Figure 1). Here we present data suggesting a novel tertiary interaction in M1 RNA between the nucleotides at positions 121 and 236. Although this interaction is not absolutely required for catalysis, our data demonstrated that a disruption resulted in a decrease in cleavage efficiency (k_{cat}/K_m). We will discuss the possibility that this interaction could be important for interaction between the RNA subunit and protein subunit of RNase P.

MATERIALS AND METHODS

Plasmid constructions and preparation of substrate and enzyme

The various M1 RNA derivatives were constructed using the polymerase chain reaction [PCR (7)] where one of the oligonucleotides carried an A to G substitution at position 121 in M1 RNA. As template we used pNL3100 (8) or a derivative of pNL3100 which carried a U to C change at position 236 in the gene encoding M1 RNA (Kirsebom, unpublished results). The resulting PCR-amplified gene(s) was cut with *Ban*II and *Sma*I, and ligated into pNL3100 (digested with the same enzymes) together with an *Kpn*I-*Ban*II fragment derived from pNL3100 which includes the remaining part of the M1 RNA gene and the promoter. To replace the wild-type promoter with the T7 promoter, the different mutant M1 RNA genes were cut with *Ban*II and *Sma*I whereas the plasmid pJA2 [which carries the wild-type M1 RNA gene behind the T7 promoter (9)] was cut with *Kpn*I and *Ban*II. The so obtained fragments were ligated into pJA2 which had been digested with *Kpn*I and *Sma*I. The different gene constructs were sequenced by the method of Sanger (10) to verify their primary structures. Transcription of these various M1 RNA genes with DNA dependent T7 RNA polymerase was performed as described elsewhere (9, 11).

Purification of the C5 protein was performed according to Vioque *et al.* (9).

The construction and generation of the precursors to $tRNA^{Tyr}$ Su3 and $tRNA^{His}$[UAG] has been described elsewhere (12).

In vivo complementation analysis

The various mutant M1 RNA derivatives cloned into pNL3100 (8) were transformed into BL21[DE3]*rnpA*49 by standard procedures. This *rnpA*49 derivative was constructed by

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generalized P1 transduction using NHY322 (13) as donor and BL21[DE3] (14) as recipient, selecting for Tet^R and screening for temperature sensitivity. The resulting strain was designated ULK136. Similarly, the strain DW2/pDW160 (15) was transformed with the same pNL3100 derivatives according to standard procedures. Growth on LB plates at the indicated temperatures was studied. The DW2/pDW160 strain was kindly provided by Dr N. R. Pace.

Northern blot analysis was performed as described elsewhere (12).

RNase P assay

The RNase P and M1 RNA activities were monitored as described elsewhere (2, 9, 16, 17). M1 RNA was preincubated in 50 mM Tris−HCl pH 7.5; 100 mM NH_4Cl; 100 mM $MgCl_2$; 5% (w/v) polyethylene glycol 6000 in absence of C5 (concentration of M1 RNA 0.02 pmol/μl) and in 50 mM Tris−HCl pH 7.5; 100 mM NH4Cl; 10 mM $MgCl_2$ in the presence of C5 (concentration of M1 RNA 0.0016 pmol/μl) for 6 min at the indicated temperature. The substrate was preincubated for 2 min at the reaction temperature before the reaction was started by mixing enzyme and substrate. The kinetic measurements were performed in the linear range of the cleavage reaction for the different substrate concentrations used under the various experimental conditions. The precursor and the cleavage products were separated on 8% denaturing polyacrylamide gels and the extent of cleavage was calculated using a phosphorimager (Molecular Dynamics 400S).

A qualitative analysis of the interaction between the various M1 RNA derivatives and C5 was performed in the following way. M1 RNA (0.0016 pmol/μl, for each M1 RNA variant studied) was preincubated in standard reaction buffer (50 mM Tris−HCl pH 7.5; 100 mM NH4Cl; 10 mM $MgCl_2$) for 5 min at 37°C. Precursor tRNA (in this case the $tRNA^{His}$[UAG] precursor; 0.056−0.067 pmol/μl) was added and the incubation was continued for 2 min. Under these conditions no cleavage by M1 RNA alone was observed. C5 protein was then added. Samples were withdrawn at different time-points after addition of C5 and the cleavage products were separated on a 10% (w/v) polyacrylamide gel in 7 M Urea and TEB-buffer [90 mM Tris-borate pH 8.5; 2.5 mM EDTA]. The extent of cleavage as percentage of total tRNA precursor added was calculated using a phosphorimager (Molecular Dynamics 400S) and plotted as a function of the time after addition of C5. As a control we added Mg^{++} (to a final concentration of 100 mM) and polyethylene glycol 6000 (to a final concentration of 5%) instead of C5.

Lead cleavage of M1 RNA

M1 RNA was 3′-end labelled with [^{32}P]pCp under standard conditions (18) and purified on an 8% denaturing polyacrylamide gel. Labelled renatured M1 RNA was cleaved with Pb^{++} following the method of Ciesiolka *et al.* (Dr R.K. Hartmann, personal communication). M1 RNA (2 pmol) was preincubated for 10 min at 37°C or 55°C in 83 mM glycine-NaOH pH 7.5, 16 mM $MgCl_2$, 167 mM NH_4Cl buffer. Pb^{++} cleavage was initiated by the addition of freshly prepared $Pb(OAc)_2$ to final concentrations of 0.5, 1.0 or 2.0 mM. This adjusted the buffer conditions to 50 mM glycine-NaOH pH 7.5, 10 mM $MgCl_2$ and 100 mM NH_4Cl. The reaction was terminated by the addition of an equal volume of 9 M Urea/50 mM EDTA/0.1% bromophenol blue after 15 min at 37°C and 5 min at 55°C. The cleavage products were separated on an 8% denaturing polyacrylamide gel. The Pb^{++} induced cleavage sites were mapped by primer-extension analysis according to Stirling *et al.* (19) using two oligodeoxynucleotide primers; one complementary to positions 166 through 190 and the other to positions 218 through 235 in M1 RNA.

RESULTS

Evidence for interaction between A121 and U236 in M1 RNA *in vivo*

Phylogenetic studies of eubacterial RNase P RNA have demonstrated covariation of the nucleotides at the positions corresponding to 121 and 236 in *E.coli* M1 RNA (5). RNase P RNA derived from *E.coli* harbours an adenosine and a uridine at these two positions, respectively (Fig. 1). To investigate whether the nucleotides at these two positions form a tertiary interaction in M1 RNA, we constructed mutants which carried substitutions at 121 and/or 236 (see Materials and Methods) and performed the following experiment.

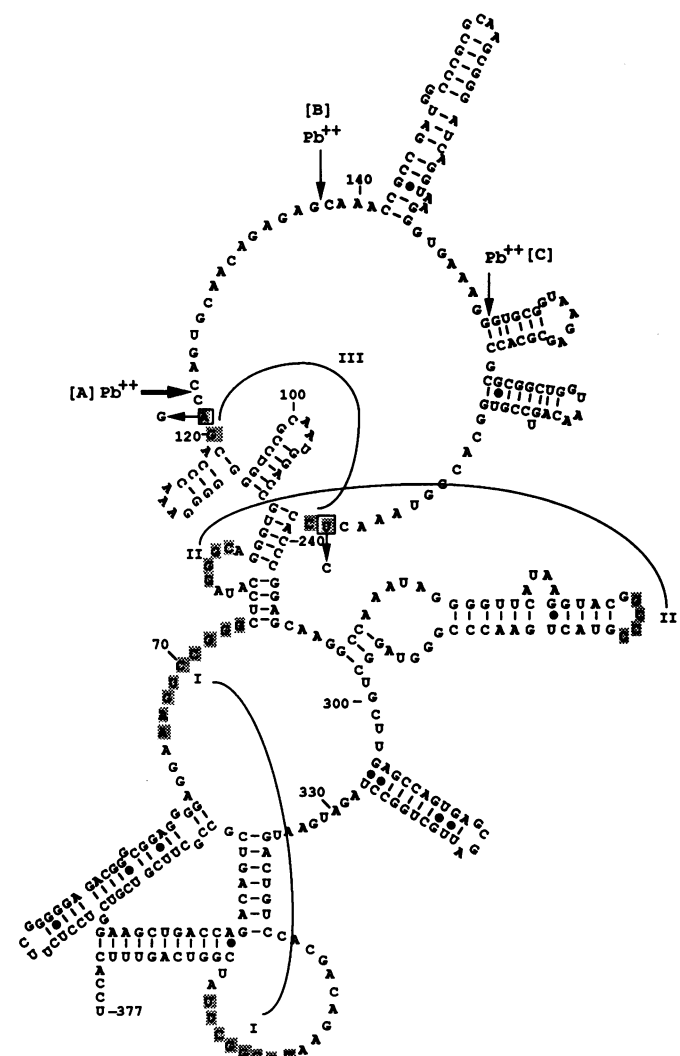

Figure 1. The secondary structure of M1 RNA according to Brown and Pace (5). The various mutations at positions 121 and 236 are indicated. The shaded nucleotides are those involved in long-range tertiary interactions (solid lines I, II and III). The Pb^{++} induced cleavage sites [A], [B] and [C] are indicated with arrows where [A] is the major cleavage site.

A mutation in *rnpA*, the gene encoding the C5 protein, results in temperature sensitivity (ts) [*rnpA*49 (3, 13)]. The ts-phenotype of a strain which harbours the *rnpA*49 allele is complemented by an excess of wild-type or certain mutant M1 RNAs (20, 21; Table 1), in particular, those mutant M1 RNAs where a disrupted base pair is restored by a suppressor mutation within the M1 RNA gene (6). When we introduced plasmids carrying the gene encoding $M1_{G121}$ RNA or $M1_{C236}$ RNA no growth at 43°C was observed (Table 1). The finding that excess $M1_{G121}$ RNA did not complement the ts-phenotype associated with *rnpA*49 was in keeping with previous reports (21). By contrast, the double mutant ($M1_{G121C236}$ RNA) resulted in complementation of the ts-phenotype (Table 1). These results suggested an interaction between the nucleotides at positions 121 and 236 in M1 RNA

Table 1. Complementation of *rnpA*49 and of a chromosomal deletion mutant by different *rnpB* alleles. Growth was analysed on LB plates at the indicated temperatures. For details see Materials and Methods.

rnpB allele	Complementation *rnpA*49 30°C	*rnpA*49 43°C	*rnpB* deletion* 30°C	*rnpB* deletion* 43°C
rnpB⁺	+	+	+	+
*rnpB*C236	+	−	+	+#
*rnpB*G121	+	−	+	+#
*rnpB*G121C236	+	+#	+	+#

#The colony size at the indicated temperature was smaller when compared to the colony size observed using *rnpB*⁺.
*The strain DW2 harbours a chromosomal deletion of *rnpB*, resulting in lethality. However, introduction of a plasmid carrying *rnpB* as in DW2/pDW160 restores viability. The plasmid pDW160 is a derivative of pSC101 which is temperature sensitive for replication. Therefore, the strain DW2/pDW160 cannot grow at 43°C (15).

in vivo. To further evaluate the predicted 121:236 base pair we studied complementation of a strain (DW2/pDW160) which cannot grow at 43°C because the M1 RNA gene is carried by a plasmid which is temperature-sensitive for replication. However, the presence of a second functional M1 RNA permits growth at the non-permissive temperature (15). The DW2/pDW160 strain was transformed with plasmids harbouring the genes encoding the wild-type or the mutant M1 RNA variants used in this study. The transformants were analysed for growth at 43°C. As can be seen in Table 1, growth was observed at the non-permissive temperature irrespective of which M1 RNA variant was used. We conclude therefore that these different mutant M1 RNA molecules are functional *in vivo* together with wild-type C5 but not with C5A49. Northern blot analysis confirmed that excess wild-type or mutant M1 RNA was synthesized *in vivo* both in the *rnpA*49 derivative and in the DW2/pDW160 strain (data not shown).

Characterization of the various M1 RNA derivatives *in vitro*

To further characterize a possible interaction between A121 and U236 we generated the wild-type and the different mutant M1 RNAs using the T7 transcription system (see Materials and Methods). *In vitro* analysis using these M1 RNA variants demonstrated that cleavage of the $tRNA^{Tyr}Su3$ and $tRNA^{His}$[UAG] precursors occurred at the expected positions both in the absence and in the presence of C5 (Fig. 2, data not shown for the $tRNA^{Tyr}Su3$ precursor). These results suggested that the A121:U236 interaction is not absolutely required for cleavage *in vitro*. Determination of K_m and k_{cat} values at 37°C using the $tRNA^{His}$[UAG] precursor showed, however, that these constants were changed for the different mutant M1 RNAs (Table 2). We consider a factor of two or more as significant which is in keeping with our previous reports (12, 17, 22, 23). In the

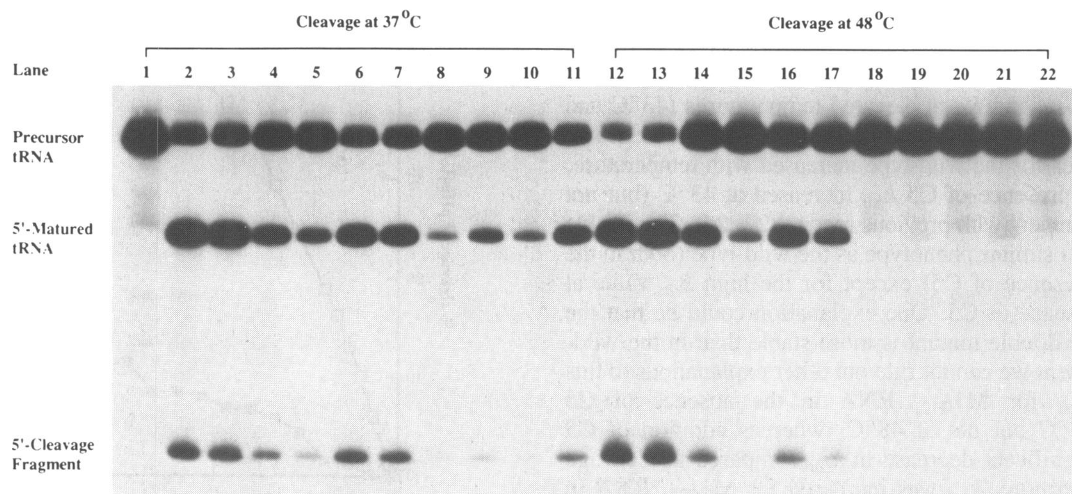

Figure 2. Processing *in vitro* of the precursor to $tRNA^{His}$[UAG] by wild-type or the various mutant M1 RNAs, in the absence and presence of C5. The experiments were performed 37°C and 48°C, and monitored as outlined in Materials and Methods. The final concentrations of reactants were as follows: precursors, 0.05 pmol/μl, M1 RNA (independent of variant) in the absence of C5 0.02 pmol/μl and 0.0016 pmol/μl in the presence of C5 (at saturating amounts). The cleavage products were separated from the precursors on a 10% denaturing polyacrylamide gel. *Lanes 1–11* cleavage performed at 37°C and *lanes 12–22* cleavage performed at 48°C. *Lane 1* = no enzyme added, time of incubation (toi) = 60 min, *lane 2* = wild-type M1 RNA, toi = 20 min, *lane 3* = $M1_{G121C236}$ RNA, toi = 20 min, *lane 4* = $M1_{G121}$ RNA, toi = 20 min, *lane 5* = $M1_{C236}$ RNA, toi = 20 min, *lane 6* = wild-type M1 RNA + C5, toi = 20 min, *lane 7* = $M1_{G121C236}$ RNA + C5, toi = 20 min, *lane 8* = $M1_{G121}$ RNA + C5, toi = 20 min, *lane 9* = $M1_{C236}$ RNA + C5, toi = 20 min, *lane 10* = $M1_{G121}$ RNA + C5, toi = 60 min, *lane 11* = $M1_{C236}$ RNA + C5, toi = 60 min, *lane 12* = wild-type M1 RNA, toi = 20 min, *lane 13* = $M1_{G121C236}$ RNA, toi = 20 min, *lane 14* = $M1_{G121}$ RNA, toi = 20 min, *lane 15* = $M1_{C236}$ RNA, toi = 20 min, *lane 16* = wild-type M1 RNA + C5, toi = 20 min, *lane 17* = $M1_{G121C236}$ RNA + C5, toi = 20 min, *lane 18* = $M1_{G121}$ RNA + C5, toi = 20 min, *lane 19* = $M1_{C236}$ RNA + C5, toi = 20 min, *lane 20* = $M1_{G121}$ RNA + C5, toi = 60 min, *lane 21* = $M1_{C236}$ RNA + C5, toi = 60 min and *lane 22* = no enzyme added, toi = 60 min.

Table 2. Kinetic constants of the RNase P reaction for the various M1 RNA derivatives in the absence and presence of C5 using the $tRNA^{His}$[UAG] precursor as substrate. The experiments were performed at 37°C, 43°C and 48oC as outlined in Materials and Methods.

Enzyme	37°C k_{cat}#	K_m#	$k_{cat}/K_m \times 10^{-6}$	43°C k_{cat}	K_m	$k_{cat}/K_m \times 10^{-6}$	48°C k_{cat}	K_m	$k_{cat}/K_m \times 10^{-6}$
M1wt RNA	2.0	95	21	4.5	234	20	8.4	250	34.3
M1G121 RNA	0.94	190	5.2	2.2	313	7.1	1.2	161	7.5
M1C236 RNA	0.18	78	2.3	0.77	200	4.0	0.40	864	0.5
M1G121C236 RNA	1.2	183	6.7	13.8	769	19	8.4	512	19.5
M1wt RNA +C5	13.5	53	259	35.8	128	278	17	110	220
M1G121 RNA +C5	17.7	123	149	5.3	80	70	ND	ND	ND
M1C236 RNA +C5	1.1	43	24	1.9	111	17.5	ND	ND	ND
M1G121C236 RNA +C5	19.3	206	93.2	26	141	186	7.9	91.4	101

#k_{cat} (pmol of substrate cleaved/min/pmol of enzyme); K_m (nM). The numbers given are based on three to four independent experiments. ND = not determined.

absence of C5 we observed reduced k_{cat} values for the mutant M1 RNA derivatives, and this was particulary pronounced for the mutant which carried a single base substitution at position 236 ($M1_{C236}$ RNA). k_{cat} using $M1_{C236}$ RNA was also significantly lowered in the presence of C5. The K_m values for the mutant M1 RNA variants, which harboured a G at position 121, were increased at 37°C compared to the wild-type, while K_m for $M1_{C236}$ RNA was unchanged, both in the absence and presence of C5.

A guanosine at position 121 generates a potential G:U base pair between positions 121 and 236. This may explain why $M1_{G121}$ RNA and the double mutant (which generates a potential G:C base pair between these positions) showed similar phenotypes with respect to the kinetics of cleavage at 37°C. An increase in reaction temperature should affect the $M1_{G121}$ RNA and the double mutant ($M1_{G121C236}$ RNA) differently, since a G:U base pair is expected to be less stable than a G:C base pair. Hence, we determined k_{cat} and K_m at elevated temperatures (43°C and 48°C) and the results are shown in Table 2. In the absence of C5 the k_{cat} values for the wild-type increased with temperature, whereas in the presence of C5 k_{cat} increased at 43°C (but not at 48°C) in agreement with previous reports (22, 24). The double mutant showed a similar phenotype as the wild-type (both in the absence and presence of C5) except for the high k_{cat} value at 43°C in the absence of C5. One explanation could be that the interation in the double mutant is more stable than in the wild-type, but at present we cannot rule out other explanations to this observation. k_{cat} for $M1_{G121}$ RNA in the absence of C5 increased at 43°C but not at 48°C, whereas addition of C5 resulted in a significant decrease in k_{cat} compared to cleavage at 37°C. Furthermore, k_{cat} was increased for $M1_{C236}$ RNA in the absence of C5 (but not in its presence) at 43°C but not at 48°C (Table 2). In the presence of C5 we observed that the single mutants ($M1_{G121}$ RNA and $M1_{C236}$ RNA) cleaved the $tRNA^{His}$[UAG] precursor with a dramatically reduced activity at 48°C (Fig. 2, lanes 19 through 22), although we did not determine the kinetic constants for cleavage at this temperature. Thus, an increase in reaction temperature showed that the phenotype of $M1_{G121}$ RNA was different relative to the phenotype of the double mutant. We also note a deterioration in the interaction between the enzyme and the $tRNA^{His}$[UAG]

Table 3. Lag-times for the interaction between M1 RNA and the C5 protein using the various M1 RNA derivatives used in this study. The experiments were performed at 37°C as outlined in Materials and Methods. The average times of several independent measurements are shown. Experimental errors given are based on 6# or 3## independent experiments, respectively.

M1 RNA	Lag-time in seconds
Wild-type M1 RNA#	31.5±31%
M1G121 RNA##	54±44%
M1C236 RNA#	130±28%
M1G121C236 RNA##	26±14%

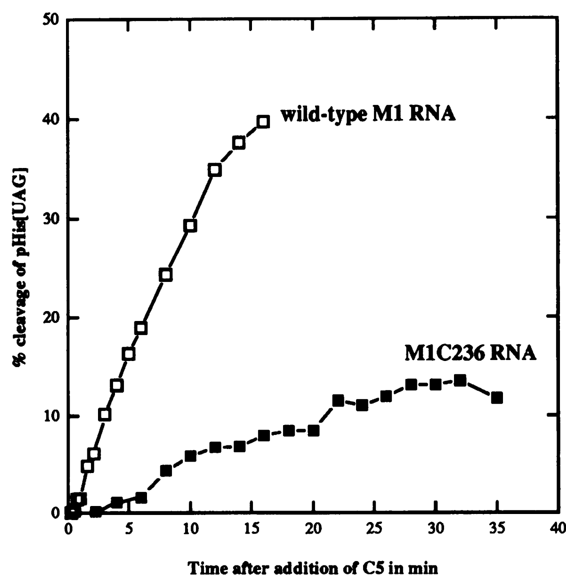

Figure 3. A qualitative analysis of the interaction between the C5 protein and wild-type or $M1_{C236}$ RNA at 37°C as outlined in Materials and Methods. Samples were withdrawn at the indicated time-intervals and the cleavage products and the precursors were separated on 10% denaturing polyacrylamide gels. The percent cleavage was calculated using a phosphorimager (Molecular Dynamics 400S) and plotted as a function of time. The lag times for the various M1 RNA derivatives used in this study are given in seconds in Table 3. Open squares = wild-type M1 RNA and filled squares = $M1_{C236}$ RNA.

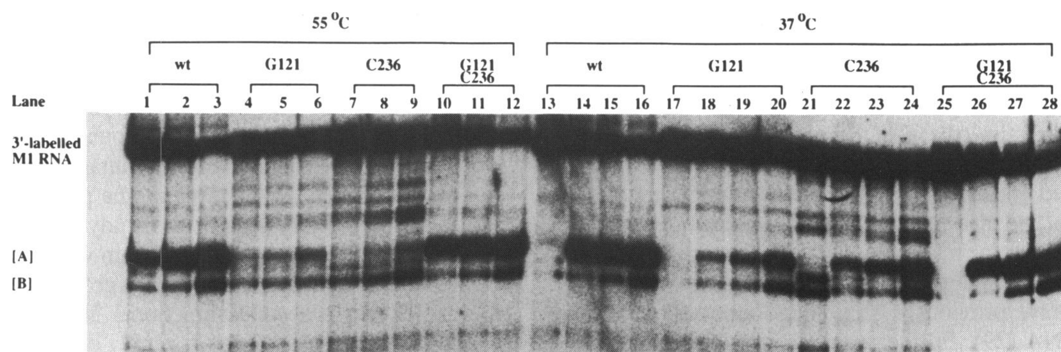

Figure 4. Lead cleavage of the various M1 RNA derivatives used in this study. Cleavage was performed at the indicated temperatures as outlined in Materials and Methods. The cleavage products and uncleaved M1 RNA were separated on an 8% denaturing polyacrylamide gel. [A] = cleavage between positions 122 and 123 and [B] = cleavage between positions 138 and 139. *Lanes 1 to 12* reaction performed at 55°C and *lanes 13 to 28* reaction performed at 37°C. *Lanes 1–3*: wild-type M1 RNA and $[Pb^{++}]$ = 0.5 mM, 1 mM and 2 mM, respectively; *lanes 4–6* $M1_{G121}$ RNA and $[Pb^{++}]$ = 0.5 mM, 1 mM and 2 mM, respectively; *lanes 7–9* $M1_{C236}$ RNA and $[Pb^{++}]$ = 0.5 mM, 1 mM and 2 mM, respectively; *lanes 10–12* $M1_{G121C236}$ RNA and $[Pb^{++}]$ = 0.5 mM, 1 mM and 2 mM, respectively; *lane 13* = wild-type M1 RNA no lead added; *lanes 14–16* = wild-type M1 RNA and $[Pb^{++}]$ = 0.5 mM, 1 mM and 2 mM, respectively; *lane 17* = $M1_{G121}$ RNA no lead added; *lanes 18–20* = $M1_{G121}$ RNA and $[Pb^{++}]$ = 0.5 mM, 1 mM and 2 mM, respectively; lane 21 = M1C236 RNA no lead added; lanes 22–24 = M1C236 RNA and [Pb++] = 0.5 mM, 1 mM and 2 mM, respectively; lane 25 = M1G121C236 RNA no lead added; lanes 26–28 = M1G121C236 RNA and [Pb++] = 0.5 mM, 1 mM and 2 mM, respectively.

precursor as determined by the K_m values when the temperature was raised.

Our *in vivo* results here suggested that disruption of the A121:U236 interaction yielded an enzyme which did not function in the presence of a mutated C5 protein (*rnpA*49) at the non-permissive temperature (Table 1). Furthermore, we did not observe any (or very little) cleavage *in vitro* for the singly mutated M1 RNAs in the presence of C5 at 48°C, whereas all four M1 RNA variants were functional in the absence of C5 at this temperature (Fig. 2; Table 2). These results might indicate that base-pairing between A121 and U236 is important for the interaction between M1 RNA and C5. To further pursue this we reasoned as follows. A lag period is observed before the rate of cleavage for the reaction catalyzed by M1 RNA (both in the absence and presence of C5) becomes linear, since M1 RNA must aquire an active conformation (24, 25). If we allowed M1 RNA to fold into an active conformation prior to addition of C5, a resulting lag period should reflect the interaction between M1 RNA and the C5 protein. Therefore, if base-pairing between A121 and U236 is of significance for the interaction with C5, a longer lag time should be observed for the single mutant M1 RNAs. Thus, we determined the lag times for the interaction between the C5 protein and the different M1 RNA variants used in this study at 37°C. For details see Materials and Methods. The results are shown in Figure 3 and Table 3.

The lag time was significantly increased when $M1_{C236}$ RNA was used compared to the lag times observed for wild-type and the double mutant ($M1_{G121C236}$ RNA). In addition, no significant change was observed for $M1_{G121}$ RNA in keeping with the notion of a G121:U236 base pair. We also observed an increased lag time for $M1_{C236}$ RNA when we used as substrate the precursor to $tRNA^{Tyr}$Su3 (L.A.K. unpublished results) suggesting that a lag period exists irrespectively of the identity of the tRNA precursor. No lag time was observed for any M1 RNA species when we adjusted the conditions such that cleavage occurred in the absence of C5 [see Materials and Methods (data not shown)]. This finding suggests that all the different M1 RNAs had aquired the active conformation required for cleavage by M1 RNA alone within 5 min. From these results it appears that disruption of the A121:U236 interaction affects the interaction between M1 RNA and C5 *in vitro*.

Probing the structure of M1 RNA by Pb^{++} cleavage

Lead cleavage has been used to study the folding of $tRNA^{Phe}$ (26–28). Recently it was demonstrated that M1 RNA is cleaved by Pb^{++} at least at two positions, 3′ to C122 and 3′ to G137 (Dr R.K. Hartmann, personal communication, see also Fig. 1). Thus, to further elucidate the suggested A121:U236 interaction we 3′-end labelled the wild-type and mutant M1 RNAs and cleaved these with Pb^{++} as outlined in Materials and Methods. These results are shown in Figure 4. The cleavage sites are indicated in Figure 1, [A] to [C].

As previously observed, wild-type M1 RNA was cleaved between C122 and C123 (the major cleavage site, denoted [A] in Fig. 1), and between G137 and C138, [B]. However, we also observed and mapped a minor cleavage site 3′ to position G184, [C]. The double mutant ($M1_{G121C236}$ RNA) was cleaved at the same positions as wild-type M1 RNA, whereas a significant reduction in the cleavage of the single mutants between positions 122 and 123 (A) was observed, in particular when cleavage was performed at 55°C. Taken together, these results suggest that Pb^{++} binds in the vicinity of position 122 and that an alteration in the local conformation near this position changes the ability of Pb^{++} to induce cleavage. This finding adds further support to the existence of an interaction between positions 121 and 236 in M1 RNA.

DISCUSSION

A novel tertiary interaction in M1 RNA

Covariation of the nucleotides at positions 121 and 236 in RNase P RNA has been demonstrated by phylogenetic analysis of eubacterial RNase P RNAs (5). This together with the *in vivo* and *in vitro* results reported here, using mutant M1 RNA variants harbouring changes at these positions, suggests the existence of a novel tertiary interaction between the nucleotides at positions

121 and 236 in *E.coli* RNase P RNA (III, see Fig. 1). This is also strongly supported by the finding that disruption of the predicted base-pairing resulted in a significant reduction in the Pb^{++} cleavage rate 3′ to position 122 in M1 RNA (this report). Phylogenetic analysis of eubacterial RNase P RNA also demonstrated that the nucleotides at positions 120 and 237 are strictly conserved [numbering refers to the numbering in M1 RNA (5)]. Thus, we also propose that the nucleotides at these two latter positions form a base pair resulting in a tertiary interaction of two base pairs. Tertiary interactions involving only two base pairs have previously been suggested to exist also in 16S and 23S rRNA (29). Furthermore, the nucleotides near the corresponding positions in several eukaryotic RNase P RNAs can form potential base pairs (30, 31). Therefore it is conceivable that a similar tertiary interaction is also present in eukaryotic RNase P RNAs.

As shown here, the A121:U236 interaction is not necessary for cleavage activity. However, our data demonstrated its importance for the efficiency of cleavage, since we observed a reduction in k_{cat}/K_m as result of a disruption of the suggested A121:U236 base pair. Recently we reported that a rate-limiting step in the cleavage reaction of the precursor to $tRNA^{Tyr}Su3$ by M1 RNA alone is product release (17). Our unpublished observations suggest that product release is not a rate-limiting step in cleavage of the $tRNA^{His}$[UAG] precursor (Tallsjö and Kirsebom, manuscript in preparation). This suggests that the k_{cat} values reported here mainly reflect the rate constant of cleavage of the phosphodiester bond. The U to C substitution at position 236 disrupts the suggested base-pairing between the nucleotides at positions 121 and 236. This generated an enzyme which cleaved the precursor to $tRNA^{His}$[UAG] with a reduced k_{cat} and an unchanged K_m. By contrast, the k_{cat} values for wild-type and $M1_{G121C236}$ RNA were essentially the same. Consequently, the A121:U236 interaction appears to be important for the actual rate of cleavage of the phosphodiester bond. Furthermore, our interpretation of the K_m values reported here (in particular at 37°C) is that base-pairing between A121 and U236 does not seem to influence the enzyme-substrate interaction to any significant extent. Rather the identity of the nucleotide at 121 appears to be important for this process (see also 21 and below).

Base-pairing between A121 and U236 may be important for interaction with C5

Disruption of the A121:U236 interaction resulted in an enzyme which in the presence of C5 was less active, in particular at 48°C, whereas in the absence of the protein we still observed significant cleavage at this temperature. The M1 RNA variant in which base-pairing between nucleotides 121 and 236 was restored ($M1_{G121C236}$ RNA) cleaved the $tRNA^{His}$[UAG] precursor with similar kinetics as wild-type M1 RNA at the higher temperatures both in the absence and presence of C5. Furthermore, the kinetic constants for cleavage of the $tRNA^{His}$[UAG] precursor by the double mutant and by $M1_{G121}$ RNA were approximately the same at 37°C but differed at elevated temperatures. This latter finding is most likely due to the formation of a G121:U236 base pair in $M1_{G121}$ RNA which is less stable at the elevated temperatures. We also observed a longer lag period at 37°C for $M1_{C236}$ RNA, compared to the wild-type, when C5 was added after M1 RNA had been allowed to aquire an active conformation (Table 3). One interpretation is that the observed lag period reflects the interaction between M1 RNA and C5, and it is therefore conceivable that disruption of the A121:U236 base pair affects this interaction.

Neither of the singly mutated M1 RNA molecules, when present in excess, complemented the *rnpA*49 temperature-sensitive phenotype, whereas the double mutant and wild-type did. The *rnpA*49 strain harbours a mutation in the gene encoding the C5 protein (3, 13). However, the presence of either of the M1 RNA derivatives used in this study permitted growth at the non-permissive temperature in a strain which carries a wild-type C5 protein. This latter strain cannot grow at this temperature in the absence of a second, functional M1 RNA (15). Previous experiments suggest that the assembly of C5A49 protein and M1 RNA is defective (24). Therefore, it is conceivable that interaction between C5A49 and M1 RNA molecules which are disrupted in the A121:U236 interaction is affected to such a degree that no growth is observed at 43°C (Table 1). By contrast, the interaction between wild-type C5 and the singly mutated M1 RNAs is not inhibited to such extent that growth at the non-permissive temperature is abolished. This interpretation is consistent with the observed prolonged lag period for $M1_{C236}$ RNA *in vitro* (see above). In conclusion, from the combined data it appears that the A121:U236 interaction is important for the interaction between M1 RNA and C5. In this context we note that breaking of a pseudoknot consisting of two base pairs by the introduction of a single base substitution in the large subunit rRNA in yeast severely reduced binding of the ribosomal protein L25 (29).

Probing the structure of M1 RNA using lead

Recently it was demonstrated that RNase P RNA is cleaved by Pb^{++} at two specific positions (Dr R.K. Hartmann, personal communication). Here we utilized this finding to probe the structure of M1 RNA which carried base-substitutions at positions 121 and/or 236. Disruption of the interaction between A121:U236 resulted in a significant reduction of Pb^{++} cleavage between positions 122 and 123. Thus, we conclude that a tertiary interaction between the nucleotides at these positions is important for Pb^{++}-induced cleavage. This result strongly supports the existence of base-pairing between A121 and U236 in M1 RNA.

It has been demonstrated that M1 RNA is specifically cleaved between positions 120 and 121 by Mg^{++}, suggesting the presence of a Mg^{++} binding site in the vicinity of these two positions (32). In the structure of $tRNA^{Phe}$ it has been shown that binding of Pb^{++} displaces Mg^{++} (33). Furthermore, the Mg^{++}-induced cleavage between positions 120 and 121 was selectively inhibited when the temperature was raised above 55°C (32). Therefore, it is conceivable that the binding sites for Mg^{++} and Pb^{++} overlap and thus base-pairing between nucleotides A121 and U236 could also be important for binding of Mg^{++}. When Mg^{++} is bound, this could result in a stabilization of the interaction between A121 and U236. Kazakov and Altman (32) discussed the possibility that the magnesium ion bound in the vicinity of A121 is involved in maximizing the initial binding of the substrate. This is in keeping with the observed increase in K_m as a result of an A to G change at this position (21; this report).

It is well-known that Pb^{++} cleaves tRNA. The best studied tRNA is yeast $tRNA^{Phe}$ and the folding of this tRNA is important for binding and cleavage with Pb^{++} (26, 33). Substitution of nucleotides involved in tertiary interactions results in a reduction in the cleavage rate, whereas normal rates were

observed when changes in nucleotides which maintain the correct folding of yeast tRNA^{Phe} were introduced (27). Furthermore, Streicher *et al.* (34) demonstrated that self-splicing group I introns were cleaved by Pb^{++} and that the Pb^{++} cleavage patterns for wild-type and a mutant T4 phage *td* intron were different. Together with the results reported here, these findings suggest that Pb^{++} cleavage could be a useful method to probe the structure of not only tRNA but also of other RNA molecules.

In conclusion, previously identified tertiary interactions in M1 RNA (I and II, see Fig. 1) add constraints to the folding of the lower half of the molecule (5, 6, 35). The presently described interaction (III, see Fig. 1) adds further constraints for the folding of M1 RNA, in particular in the upper half of the molecule.

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