A Physical Assay for and Kinetic Analysis of the Interactions between M1 RNA and tRNA Precursor Substrates†

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ABSTRACT: A gel–shift assay was devised to detect stable enzyme–substrate (E–S) complexes between M1 RNA, the catalytic subunit of RNase P from *Escherichia coli*, and its tRNA precursor substrates. The use of deletion derivatives of M1 RNA in the gel–shift assay has allowed us to identify regions of the enzyme that are involved in the binding of the substrate or that are necessary for catalytic activity. Fragments of substrates that contain the 3′ CCA sequence bind preferentially to regions in the 5′ half of M1 RNA, while 5′ leader sequences interact primarily with regions in the 3′ half of M1 RNA. The 5′ leader sequence present in the precursor to $tRNA^{Tyr}_{su3}$ from *E. coli* plays an important role in the formation of stable E–S complexes with M1 RNA. The CCA sequence at the 3′ end of precursor tRNA substrates is involved in the product-release step of the reaction that is catalyzed by M1 RNA. Direct measurements of the concentrations of all the components in the reaction catalyzed by M1 RNA facilitated a new approach to the kinetic analysis of the action of the enzyme.

The catalytic activity of RNase P from *Escherichia coli* resides in the RNA subunit (M1 RNA) of the enzyme (Guerrier-Takada et al., 1983). Previous studies with derivatives of M1 RNA (Guerrier-Takada & Altman, 1992; Guerrier-Takada et al., 1989; Kirsebom & Altman, 1989) indicated that cleavages occur at different rates with different substrates. This observation and the results of both physicochemical and genetic studies (Guerrier-Takada & Altman, 1992; Knap et al., 1990; Lumelsky & Altman, 1988) suggested that M1 RNA is flexible and acquires, in a complex fashion, an active conformation that is specific for the substrate to be cleaved. Efforts at understanding the mechanism of action of RNase P have, however, been limited to analysis of the kinetics of the assay (i.e., cleavage of ptRNA[1] substrates) that is used to measure the catalytic activity. We have now developed a gel–shift assay that allows us to detect stable enzyme–substrate (E–S) complexes between M1 RNA and certain substrates and to calculate the kinetic constants for various steps in the specific cleavage reactions; the values obtained are similar to those reported previously from standard assays of enzymatic activity. Using this new assay, we have also been able to determine binding constants between various RNA molecules in solution.

We show here that the interactions of M1 RNA with segments of a tRNA precursor (ptRNA) substrate can be localized as follows: the 3′ half of the substrate (containing the 3′ terminal CCA sequence) binds preferentially to regions in the 5′ half of the M1 RNA, and the 5′ portion of the substrate (containing the 5′ leader sequence) binds preferentially to regions in the 3′ half of M1 RNA. The 3′ terminal CCA sequence governs the interactions in the 3′ half of the substrate. By contrast, in the 5′ portion of the substrate, the nature of the interactions is much less clear, in part because there are no conserved sequences or nucleotides. Accordingly, we have made an attempt to determine whether certain chemical features of, or groups of nucleotides in, the 5′ leader sequences are the determining factors in recognition by M1 RNA of its substrates.

MATERIALS AND METHODS

Materials. Nucleoside triphosphates were purchased from Pharmacia P-L Biochemicals, [α-^{32}P]GTP (410 Ci/mmol) from Amersham Biochemicals, T7 RNA polymerase and SP6 RNA polymerase from Promega Biotec, and restriction enzymes from New England Biolabs. All chemicals were of reagent grade.

Preparation of RNA. Plasmids encoding derivatives of M1 RNA were linearized with the appropriate restriction endonuclease and transcribed by T7 RNA polymerase as previously described (Vioque et al., 1988). RNA substrates were labeled with [α-^{32}P]GTP during transcription *in vitro* and purified on denaturing polyacrylamide gels. The derivatives of M1 RNA (Guerrier-Takada & Altman, 1992) are indicated by the symbol Δ followed by numbers between square brackets; these numbers indicate the extent of deletion within the nucleotide sequence of M1 RNA (for example, Δ[156–377]; see Figure 1). Plasmid encoding the precursor to $tRNA^{Ser}$ from yeast (pSupS1) was a gift from Drs. D. Söll and I. Willis (Yale University).

Assays of RNase P Activity. Cleavage of RNA substrates was performed with RNase P (the holoenzyme) in 50 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 10 mM $MgCl_2$ (buffer A). M1 RNA and its derivatives were assayed in buffer A supplemented with 90 mM $MgCl_2$ and 4% PEG[2] (buffer B).

Binding Assays. M1 RNA or its derivatives and RNA substrates (or oligonucleotides[3]) were incubated together in 10 μL of buffer B for 10 min at 37 °C. One microliter of 10× loading dye (50% glycerol and 0.5% brom phenol blue) was

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[1] Abbreviations: ptRNA, tRNA precursor; p43Tyr, precursor to $tRNA^{Tyr}$ from *Escherichia coli*; p15Phe, precursor to $tRNA^{Phe}$ from *E. coli*; pSer, precursor to human $tRNA^{Ser}$; pSupS1, precursor to $tRNA^{Ser}$ from yeast; pAva, truncated p43Tyr RNA transcript that contains the 5′ leader sequence followed by the first 15 nucleotides within the mature $tRNA^{Tyr}$ sequence; tTyr, $tRNA^{Tyr}$ from *E. coli*; $oligo_{(x-y)}$, DNA oligonucleotide complementary to M1 RNA, where x and y indicate the range, within the nucleotide sequence of M1 RNA, to which the oligonucleotide is complementary.

[2] Poly(ethylene glycol).

[3] DNA oligonucleotides.

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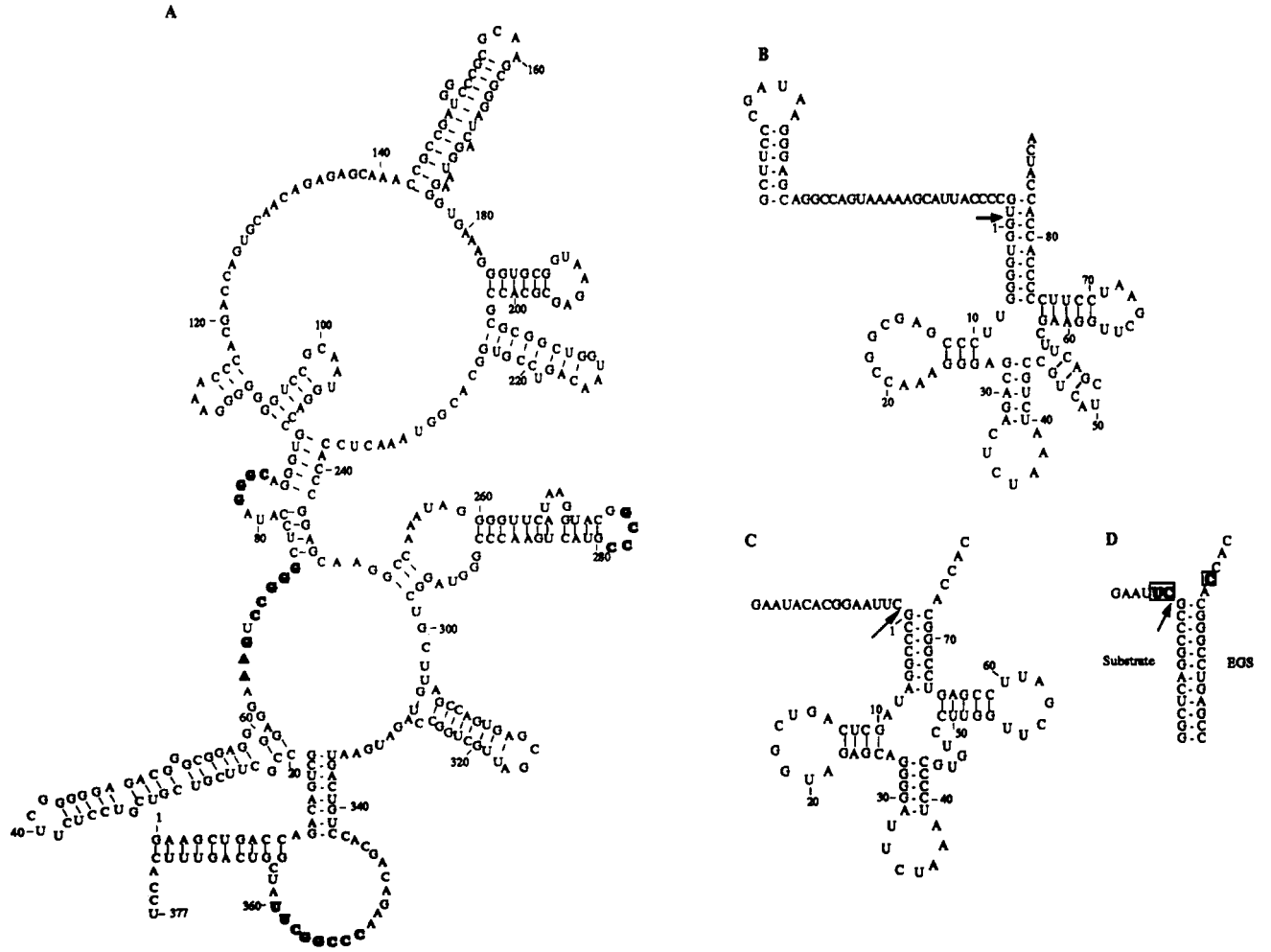

FIGURE 1: Schematic representation of the secondary structure of M1 RNA (A; James et al., 1988) and the secondary structures of several substrates for RNase P (B, C, D). (B) The precursor to $tRNA^{Tyr}_{su3}$ (p43Tyr) from *E. coli*; (C) the precursor to $tRNA^{Phe}$ (p15Phe) from *E. coli*; (D) model substrate, derived from $tRNA^{Phe}$ (p15Phe) from *E. coli*, composed of two oligoribonucleotides, with the external guide sequence (EGS) indicated. The 2′-OH groups of the boxed and bold nucleotides are important for efficient cleavage of the substrate by M1 RNA. The arrows indicate the site of cleavage by RNase P.

added, and the sample was then loaded on a 3% agarose gel prepared with 50 mM Tris-borate, pH 8.3, 1 mM $MgCl_2$. If an annealing step was necessary, the enzyme and the RNA substrate (or oligonucleotide) were incubated together, in buffer A, at 65 °C for 5 min and slowly cooled to room temperature prior to the 10-min incubation described above. When both samples were unlabeled, ethidium bromide (0.5 μg/mL) was added to the agarose gel and the complexes were visualized on a UV transilluminator; if a labeled substrate was used, the complex was detected by autoradiography.

Direct Determination of Binding Constants. Binding constants were determined as described previously (Pyle et al., 1990) with the following modifications: M1 RNA at different concentrations was incubated in buffer B at 37 °C for at least 10 min in the presence of a fixed concentration of 5′ end-labeled oligonucleotide. This time of incubation was found to be sufficient for the reaction to reach equilibirum. After the addition of loading dye, the samples were immediately loaded on a 3% agarose gel (0.4 × 20 × 25 cm^3) and electrophoresis was carried out at 60 mA for about 2 h. The electrophoresis buffer was the same as mentioned above. The gel was dried, and amounts of free oligonucleotide and its complex were quantified with a Betascope blot analyzer (Betagen).

Pulse–Chase Experiments. M1 RNA (10 nM) and radiolabeled p43Tyr (200 nM) substrate were incubated together in 200 μL of buffer B for 11 min at 37 °C. Three aliquots of 50 μL were then withdrawn, and each was added to 2 μL of H_2O, a solution of tRNA (12.5 pmol/μL), or a solution of oligonucleotide complementary to nucleotides −19 to −4 in the 5′ proximal sequence (15 pmol/μL); the mixture was incubated at 37 °C for a total of 40 min, and aliquots were removed at intervals and analyzed on a 3% agarose gel. Both rates of cleavage and binding efficiencies were calculated from data obtained by analysis of the gel with the Betascope.

RESULTS

Specificity of Binding. Electrophoresis on nondenaturing gel has been used to study associations not only between nucleic acids and proteins but also between different RNA molecules. Recently, the association of oligonucleotides with the group I intron derived from *Tetrahymena* rRNA has been examined with this method (Pyle et al., 1990). Previous attempts to study stable, noncovalently linked E–S complexes between M1 RNA and its ptRNA substrates (Figure 1), using nondenaturing polyacrylamide gels, were not productive because of the low efficiency of detection of the E–S complex (Altman et al., 1987). We improved this method in an attempt to distinguish derivatives of M1 RNA that were deficient in the chemical step from those deficient in the substrate-binding step of the reaction catalyzed by RNase P. The assay for formation of E–S complexes was optimized by replacing EDTA

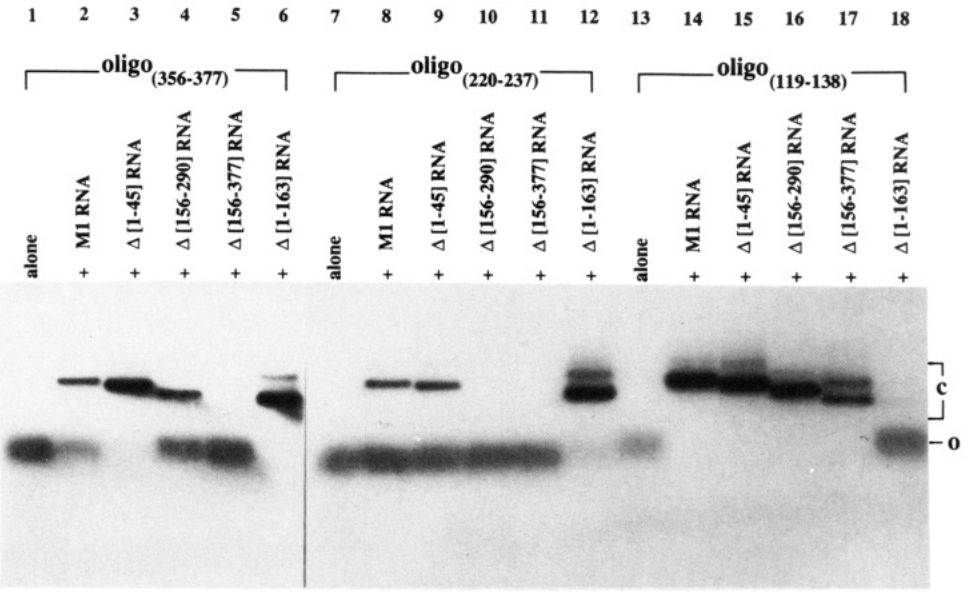

FIGURE 2: Binding of oligonucleotides to derivatives of M1 RNA. ^{32}P-labeled oligonucleotides (5 nM), complementary to nucleotides 356–377 ($oligo_{(356-377)}$), 220–237 ($oligo_{(220-237)}$), and 119–138 ($oligo_{(119-138)}$) in M1 RNA, were incubated with derivatives of M1 RNA (100 nM) as described in Materials and Methods. All samples were analyzed on a 3% agarose gel. Lanes 1–6, binding of $oligo_{(356-377)}$ to derivatives of M1 RNA; lanes 7–12, binding of $oligo_{(220-237)}$ to derivatives of M1 RNA; lanes 13–18, binding of $oligo_{(119-138)}$ to derivatives of M1 RNA. Lanes 1, 7, and 13, no RNA added; lanes 2, 8, and 14, wild-type M1 RNA added; lanes 3, 9, and 15, Δ[1–45] RNA added; lanes 4, 10, and 16, Δ[156–290] RNA added; lanes 5, 11, and 17, Δ[156–377] RNA added; lanes 6, 12, and 18, Δ[1–163] RNA added. The position of the oligonucleotides (o) and the region of the complexes detected (c) are indicated. In some lanes, dimers of derivatives of M1 RNA are visible.

Table I: Binding of Oligonucleotides to Derivatives of M1 RNA and Binding of RNA Molecules[a]

entry no.	labeled molecule	M1 RNA	unlabeled oligonucleotide	K_d (nM)
1	$oligo_{(56-74)}$	wild-type	none	50 ± 1.0
2	$oligo_{(56-74)}$	wild-type	$oligo_{(119-138)}$ (500 nM)	19 ± 3.3
3	$oligo_{(56-74)}$	wild-type	$oligo_{(347-363)}$ (500 nM)	36 ± 4.3
4	$oligo_{(56-74)}$	wild-type	$oligo_{(356-377)}$ (500 nM)	58 ± 0.7
5	$oligo_{(56-74)}$	Δ[65] RNA	none	104 ± 1.0
6	$oligo_{(56-74)}$	Δ[65] RNA	$oligo_{(119-138)}$ (500 nM)	36 ± 3.5
7	$oligo_{(56-74)}$	Δ[92] RNA	none	52 ± 2.3
8	$oligo_{(56-74)}$	Δ[92] RNA	$oligo_{(119-138)}$ (500 nM)	24 ± 3.6
9	$oligo_{(119-138)}$	wild-type	none	5 ± 2.8
10	$oligo_{(119-138)}$	Δ[65] RNA	none	10 ± 6.2
11	$oligo_{(119-138)}$	Δ[92] RNA	none	10 ± 5.7
12	$oligo_{(347-363)}$	wild-type	none	435 ± 0.1
13	$oligo_{(356-377)}$	wild-type	none	230 ± 0.1
14	p43Tyr	Δ[156–290] RNA	none	105 ± 1.0
15	p43Tyr	Δ[65] RNA	none	41 ± 0.1
16	Δ[156–377] RNA	Δ[1–163] RNA	none	15 ± 1.2

[a] ^{32}P-labeled oligonucleotides or RNA molecules (M1 RNA and its derivatives) were incubated in the presence of increasing amounts of unlabeled RNA alone or in the presence of unlabeled oligonucleotide (500 nM) as indicated. The assays were carried out as described in Materials and Methods. The values for K_d were obtained by solving the equilibrium equation at the (mid)point where $[oligo_{bound}] = [oligo_{free}]$; $K_d = [RNA]_{midpoint} - {}^1/_2[oligo_{total}]$. The error values are the standard deviations derived from calculations of the best fit of the data to a computer-generated curve in individual binding experiments (Pyle et al., 1990).

with 1 mM $MgCl_2$ in the Tris-borate running buffer of nondenaturing gels. In this low concentration of Mg ion, M1 RNA still carries out the cleavage reaction albeit inefficiently. The amount of material found in E–S complexes was independent of the duration of electrophoresis. Under these conditions, results were obtained that were consistent with those obtained by other methods, and, therefore, these observations are very likely an accurate reflection of the distribution of components in the reaction mixture prior to loading on the gels (see below).

To determine whether or not the interactions detected with this new assay were specific, we measured the binding of several oligonucleotides complementary to M1 RNA as well as the annealing between the 5′ half (Δ[156–377] RNA) of M1 RNA and the 3′ half (Δ[1–163] RNA) of M1 RNA. The two half-molecules, if they associate correctly via conventional Watson–Crick hydrogen bonding and generate a wild-type structure, should comigrate with intact M1 RNA. Our results indicated that the binding that we measured was specific, i.e., oligonucleotides bound to derivatives of M1 RNA only when the sequence to which they were complementary was present in the RNA template (Figure 2). Furthermore, annealing of the two half-molecules occurred as predicted (data not shown), and an active catalytic complex was formed (Guerrier-Takada & Altman, 1992). Indeed, no binding was observed in lane 5 in Figure 2, in which the derivative of M1 RNA that was used contained a deletion that extended into the region to which the $oligo_{(356-377)}$ is complementary; similar results can be seen in lanes 10, 11, and 18.

The region in M1 RNA between nucleotides 120 and 140 should be very accessible to complementary oligonucleotides according to a current model of the secondary structure of M1 RNA (James et al., 1988), and our results show that this prediction is borne out (Figure 2 and Table I). However, the region between nucleotides 220 and 240, also thought to be single-stranded (James et al., 1988), is relatively less accessible to binding by a complementary oligonucleotide (Figure 2, compare lane 8 with lane 14). This result indicates that the nucleotides between positions 220 and 240 either interact with another, distant part of M1 RNA or they are buried in the three-dimensional structure of the molecule. A deletion of the first 45 nucleotides at the 5′ end of M1 RNA has no effect on the binding of $oligo_{(220-237)}$ (compare lanes 8 and 9), but it improves the binding of $oligo_{(356-377)}$ considerably (compare lanes 2 and 3). This result is expected from the model for the

secondary structure of M1 RNA (James et al., 1988) because nucleotides 356–377 are not part of a hydrogen-bonded region in the deletion derivative.

From results similar to those shown in Figure 2, we have determined the dissociation constants for the interactions of several oligonucleotides with M1 RNA and some of its derivatives. The data obtained confirm that some regions of M1 RNA are more accessible to binding than others. Specifically, nucleotides 119–138 are readily available for binding while nucleotides 347–363 are much less accessible. The latter result can be explained on the basis of a phylogenetically conserved helical structure (James et al., 1988) between nucleotides 353–360. However, our data also indicate that the other region of M1 RNA that is involved in the same helical region (nucleotides 56–74) is much more accessible and, therefore, appears to be located on the "outside" of the molecule (Table I, compare entry 1 with entry 12). These latter results are consistent with a three-dimensional structure of M1 RNA generated by computer modeling techniques (E. Westhof and S. Altman, unpublished observations).

Interactions between Different Regions of M1 RNA. We used the gel–shift assay to study interactions between different regions of M1 RNA. A similar approach was recently used to study the conformation of rRNA molecules (Weller & Hill, 1992). To our surprise, the binding of an oligonucleotide complementary to nucleotides 119–138 ($oligo_{(119-138)}$) facilitates the binding of an oligonucleotide complementary to nucleotides 56–74 ($oligo_{(56-74)}$) (Table I, compare entry 1 with entry 2). The binding of an oligonucleotide complementary to nucleotides 56–74 to two derivatives of M1 RNA that contain single-nucleotide deletions Δ[65] RNA and Δ[92] RNA was also examined. As in the case of wild-type M1 RNA, the binding of $oligo_{(56-74)}$ to these derivatives improved significantly in the presence of an oligonucleotide complementary to nucleotides 119–138 (Table I, compare entry 5 with entry 6 and entry 7 with entry 8). Thus, some nucleotides in the sequence 119–138 clearly also interact with some nucleotides between 56 and 74. We note that the single-base deletion at position 65 alters the conformation of M1 RNA to make nucleotides 56–74 less accessible to the complementary oligonucleotide than they are in wild-type M1 RNA (Table I, compare entry 1 with entry 5, also, see below).

Regions of M1 RNA Involved in Interactions with Substrates. We examined several fragments of M1 RNA, all of which are catalytically inactive, to determine whether any retained the ability to bind a true substrate (p43Tyr). Our results (Figure 3, panel A) indicate that several fragments (Δ[1–45] RNA, Δ[1–163] RNA, Δ[62–108] RNA, Δ[94–204] RNA, Δ[94–290] RNA, Δ[156–290] RNA, and Δ[1–45, 273–377] RNA) are able to bind the substrate, while Δ[156–377] RNA, which lacks the entire 3′ half of M1 RNA, cannot. Thus, the features of M1 RNA that are essential for the interaction between the enzyme and the substrate are controlled mainly by sequences located in the 3′ half of the enzyme. If we considered the results obtained with all the derivatives of M1 RNA that still retain some ability to bind to the substrate, we find that no single, local region of M1 RNA is *entirely* responsible for the interaction with the substrate. Results shown in lanes 8, 9, and 10 in Figure 3A are consistent with this conclusion and indicate that the presence of the phylogenetically conserved helix (that is a part of a pseudoknot) between nucleotides 66 and 74 and 353 and 360 stabilizes the interaction between the enzyme and the substrate. These data confirm previous observations that cleavage of substrates appears to depend on interactions between the substrate and several localized regions of M1 RNA (James et al., 1988; Lumelsky & Altman, 1988; Shiraishi & Shimura, 1986). (We note that a binding constant for binding of wild-type M1 RNA to a ptRNA substrate is not included in Table I; it was not possible to separate this step in the overall reaction completely from the cleavage event when these components were used.)

Regions of a Substrate Necessary for Binding to M1 RNA. Studies on UV-cross-linked E–S complexes between M1 RNA and ptRNA substrates (Guerrier-Takada et al., 1989) indicated that a region near the site to be cleaved in the 5′ leader sequence is in close contact with M1 RNA. Furthermore, observations of the role of 2′-hydroxyl groups (Perreault & Altman, 1992) and the binding of metal ions to substrates (Kazakov & Altman, 1991) also suggested that the same regions in the 5′ leader sequence and the 3′ terminal CCA sequence (in the tRNA domain) play important roles in the recognition and cleavage of ptRNA substrates by M1 RNA. Other data have shown that tRNA is an inhibitor of the cleavage activity of RNase P (Guerrier-Takada et al., 1984; Reich et al., 1988). Accordingly, we studied the interaction of substrates with M1 RNA by examining the roles of the tRNA domain and the 5′ leader sequences separately.

If a substrate interacts with M1 RNA primarily via the tRNA domain and if the inhibition by tRNA of the cleavage reaction is due to the fact that the tRNA product binds more strongly to M1 RNA than the substrate (thereby giving the appearance of slow release of product (P) during the enzymatic reaction), E–P complexes should be detectable on agarose gels, as are E–S complexes. However, $tRNA^{Tyr}$, even though it has the 3′ terminal CCA sequence (Figure 3A), does not bind to M1 RNA or any of its tested derivatives. Clearly, structural features of ptRNAs, in addition to those of the tRNA domain (e.g., the 3′ terminal CCA sequence), must contribute to the interaction with the enzyme.

Previous experiments (Knap et al., 1990) indicated that the tertiary structure of p43Tyr unfolds when the substrate binds to M1 RNA during the cleavage reaction. These experiments did not rule out the possibility that immediately after cleavage, while still on the surface of the enzyme, the tertiary structure of the cleaved substrate (the product) remains unfolded. We now assert, however, that the mature $tRNA^{Tyr}$ product, when separated from the enzyme, has the "conventional" tRNA conformation (and, unlike a ptRNA, does not unfold during the incubation with M1 RNA). Hence, it cannot form an E–P complex (in which P represents mature tRNA in its conventional, folded conformation) sufficiently stable that it is detectable in our system. If these arguments are correct, an annealing step prior to the standard incubation for formation of complexes between $tRNA^{Tyr}$ and M1 RNA might allow the detection of an E–P complex (in which P represents mature tRNA in an unfolded conformation). Indeed, our results (Figure 3B) indicate the formation of such a complex when an annealing step is included. However, the sites for binding of substrate and (unfolded) product are not identical (although they might overlap or represent different conformational states of the enzyme), as determined by our analysis of the kinetics of inhibition of the cleavage reaction (see below). The main difference in the binding results is that the (unfolded) product binds better to the 5′ half of M1 RNA, while the substrate (which has a 5′ leader sequence in addition to the tRNA domain) prefers the 3′ half of M1 RNA (compare, in Figure 3, lanes 3 and 5 in panel A with lanes 4 and 5 in panel B).

We might expect the mechanism of interaction of p43Tyr with M1 RNA to be applicable to all ptRNA substrates. That

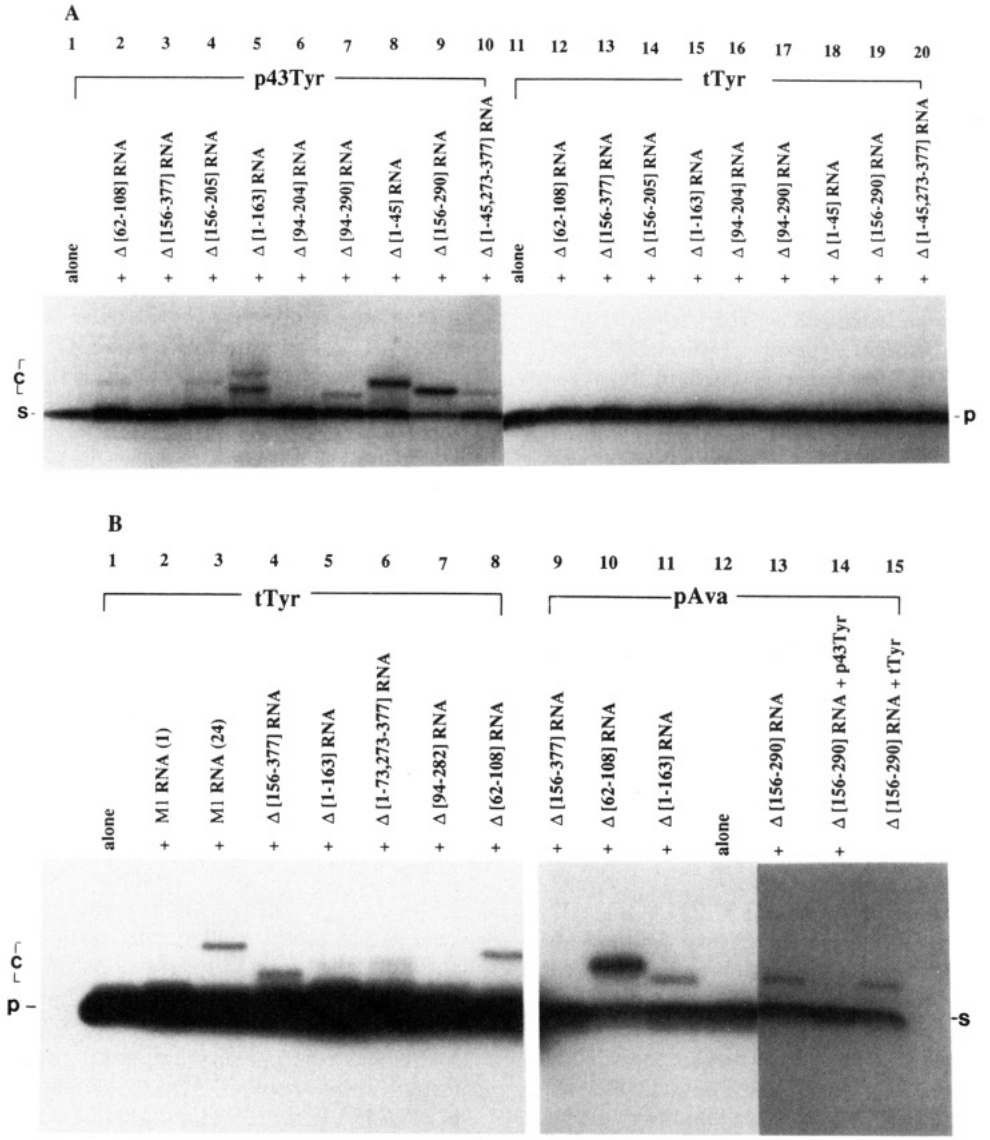

FIGURE 3: Binding of p43Tyr, tTyr, and pAva RNAs to derivatives of M1 RNA. Panel A, ^{32}P-labeled p43Tyr (lanes 1–10) or tTyr (lanes 11–20) RNA transcripts (5 nM) were incubated for 10 min at 37 °C in buffer B in the presence of several derivatives of M1 RNA (100 nM) as indicated. After addition of the loading dye solution, the samples were loaded onto a 3% agarose gel as described in Materials and Methods. Lanes 1 and 11, no M1 RNA added; lanes 2 and 12, Δ[62–108] RNA added; lanes 3 and 13, Δ[156–377] RNA added; lanes 4 and 14, Δ[156–205] RNA added; lanes 5 and 15, Δ[1–163] RNA added; lanes 6 and 16, Δ[94–204] RNA added; lanes 7 and 17, Δ[94–290] RNA added; lanes 8 and 18, Δ[1–45] RNA added; lanes 9 and 19, Δ[156–290] RNA added; lanes 10 and 20, Δ[1–45, 273–377] RNA added. Panel B, lanes 1–8, ^{32}P-labeled tTyr RNA transcript (5 nM) was annealed with derivatives of M1 RNA (100 nM) as described in Materials and Methods, and then the mixtures were incubated at 37 °C in buffer B. Lane 1, no M1 RNA added; lane 2, M1 RNA added (100 nM); lane 3, M1 RNA added (2400 nM); lane 4, Δ[156–377] RNA added; lane 5, Δ[1–163] RNA added; lane 6, Δ[1–73, 273–377] RNA added; lane 7, Δ[94–282] RNA added; lane 8, Δ[62–108] RNA added. Lanes 9–15, ^{32}P-labeled pAva RNA transcript (5 nM) was incubated with derivatives of M1 RNA (100 nM) in the absence or in the presence of p43Tyr (100 nM) or tTyr (570 nM). Lane 9, Δ[156–377] RNA added; lane 10, Δ[62–108] RNA added; lane 11, Δ[1–163] RNA added; lane 12, no M1 RNA derivative added; lane 13, Δ[156–290] RNA added; lane 14, Δ[156–290] RNA and p43Tyr added; lane 15, Δ[156–290] RNA and tTyr added. All samples were analyzed on a 3% agarose gel. The positions of the substrates (s), products (p), and complexes (c) are indicated.

is, on incubation with the enzyme there should be an unfolding of the tertiary structure of the ptRNA and formation of the E–S complex should then occur, as was inferred previously from the results of studies with mutants of M1 RNA with large deletions and pTyr (Guerrier-Takada & Altman, 1992). However, attempts to detect the formation of E–S complexes between M1 RNA and other ptRNA substrates (e.g., p15Phe, pSer, pSupS1) in our system were unsuccessful, even when an annealing step was included prior to mixing of components of the reaction and the concentration of the substrate was close to its K_M with M1 RNA. It seemed possible, therefore, that the 5′ leader sequence of p43Tyr uniquely dominates the interaction of this substrate with the enzyme in a manner that remains to be specified. Accordingly,we postulated that if this 43-mer leader sequence were added to any tRNA, detection of stable E–S complexes between the chimeric ptRNA and M1 RNA might be facilitated. We constructed a substrate in which the 5′ leader sequence of p43Tyr was ligated to $tRNA^{Phe}$ (p43Phe) and tested its ability to form an E–S complex. Our results show that, unlike p15Phe (Figure 4, lane 2), p43Phe formed a stable E–S complex with M1 RNA (lane 6), thereby demonstrating that the 5′ leader sequence of p43Tyr participates directly in an interaction with the enzyme. We also constructed a pTyr substrate (p14Tyr) with a 14-nucleotide leader sequence that was derived from the p15Phe clone (McClain et al., 1987) and followed the formation of the E–S complex. No stable E–S complex was detected between M1 RNA and p14Tyr (with or without a

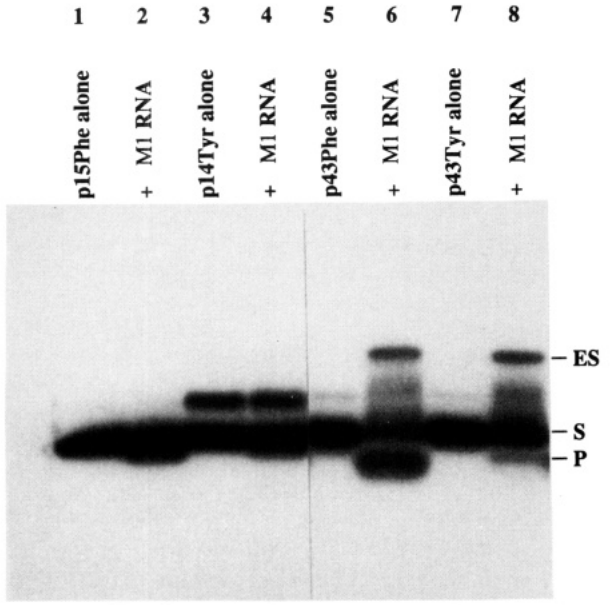

FIGURE 4: Effect of the 5′ leader sequence on the formation of stable E–S complexes. Binding assays were carried out as described in the legend to Figure 3. Lanes 1, 3, 5, and 7, ^{32}P-labeled substrate (10 nM) alone; lanes 2, 4, 6, and 8, ^{32}P-labeled substrate incubated in the presence of M1 RNA (10 nM); lanes 1 and 2, p15Phe, as the substrate; lanes 3 and 4, p14Tyr as the substrate; lanes 5 and 6, p43Phe as the substrate; lanes 7 and 8, p43Tyr as the substrate. The nature of the substrate and the positions of E–S complexes, S (substrate), and P (product) are shown. In lanes 3 and 4, a band corresponding to a dimer of the p14Tyr substrate is clearly visible, and in lanes 5 and 7 a lighter band corresponding to a dimer of the substrate can also be seen.

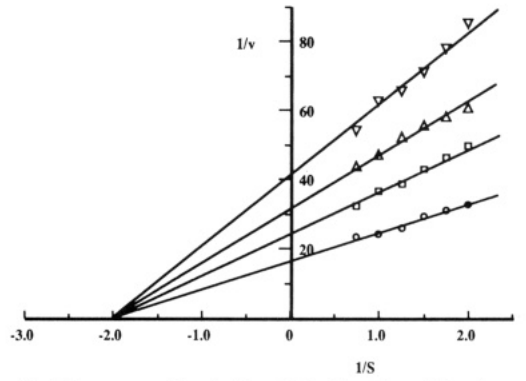

FIGURE 5: Lineweaver–Burk plot of the kinetics of the cleavage by M1 RNA of p43Tyr in the presence or absence of inhibitor. M1 RNA was incubated in buffer B for 5 min at 37 °C. Aliquots of this reaction mixture were added to ^{32}P-labeled p43Tyr (at the concentrations indicated) or to a mixture of ^{32}P-labeled p43Tyr and unlabeled tRNAs (50, 100, and 150 nM). Aliquots were removed during the linear portion of the reaction, and the products of the reaction were analyzed on an 8% polyacrylamide gel that contained 7 M urea. A Lineweaver–Burk double-reciprocal plot was constructed from the data. Units: $1/v$, 10^8 M^{-1} min; $1/S$, 10^8 M^{-1}. (○) No inhibitor added; (□) 50 nM tRNA added; (△) 100 nM tRNA added; (▽) 150 nM tRNA added.

preannealing step), an indication once again of the unique importance of the 5′ leader sequence of p43Tyr in the interaction with M1 RNA (Figure 4, lanes 4 and 8). Note that nucleotides −9 to −5 of the 5′ leader sequence of p43Tyr are complementary to nucleotides 229–233 in M1 RNA, a region of this latter molecule that is thought to be near the active site. This sequence complementarity may be responsible for the unique ability of the 5′ leader sequence of p43Tyr to stabilize the interaction between enzyme and substrate. There are additional similarities among sequences in p43Tyr and M1 RNA that might be important in E–S interactions. For example, base pairs formed between nucleotides 2–6 with 80–76 in p43Tyr are similar in sequence to base pairs formed between nucleotides 87–91 with nucleotides 242–238 in M1 RNA. Denaturation of the aminoacyl stem during binding to the enzyme may then allow base pairing of complementary nucleotides in the enzyme and substrate (see below).

To identify unequivocally those regions of p43Tyr that are involved in the interaction with the enzyme, an additional test molecule was used in the gel–shift assay. This molecule was an RNA substrate (pAva), which contained 43 nucleotides that corresponded to the 5′ leader sequence of p43Tyr, followed by 15 nucleotides within the mature $tRNA^{Tyr}$ sequence. pAva bound most strongly to RNA molecules that contain the 3′ half of M1 RNA (Figure 3B, compare lane 9 with lanes 10 and 11). It is of interest to note that p43Tyr, which binds well to Δ[156–290] RNA (Figure 3A, lane 9), competed with pAva for binding to this derivative of M1 RNA (Figure 3B, lanes 13 and 14); by contrast, tTyr (lane 15) did not. Other RNAs, as well as oligonucleotides containing the CCA sequence, bound to sequences that contained the 5′ half of M1 RNA and more specifically to a region between nucleotides 62 and 108. Indeed, the binding of these molecules was abolished if Δ[62–108] RNA, a derivative of M1 RNA, was used (data not shown).

Additional data regarding regions of substrates that are critical for interactions with M1 RNA come from studies of a derivative of a small model substrate (p6AT1), which consists of two base-paired oligonucleotides, namely the target substrate, an RNA molecule which has the first 18 nucleotides of p6-AT1, and an external guide sequence (EGS) (Forster & Altman, 1990; Perreault & Altman, 1992). The 18-mer can be cleaved by M1 RNA, in the presence of an EGS (Perreault & Altman, 1992). It also binds very well to M1 RNA, notably to its first 94 nucleotides (Δ[95–377] RNA). The 18-mer does not have a 3′ terminal CCA sequence, but there is 100% complementarity between nucleotides 67–75 within the M1 RNA sequence and nucleotides 1–9 in the 18-mer. However, binding to the enzyme is completely eliminated if the EGS is present in the reaction, an indication that the interaction between the substrate and the EGS is stronger than the interaction of the 18-mer with M1 RNA.

Unlike p43Tyr, small model substrates cannot undergo the necessary unfolding of a tRNA-like tertiary structure when they interact with M1 RNA. A derivative (pAT) of pAT1, containing a 5′ leader sequence identical to that of p43Tyr, did not form an E–S complex with M1 RNA unless an annealing step was included prior to the standard incubation. It has been reported that denaturation of the equivalent of the aminoacyl acceptor stem of a ptRNA occurs subsequent to unfolding of tertiary structure upon binding to M1 RNA (Knap et al., 1990); our results indicate that such a (denaturation) step facilitates the binding of at least one small model substrate to M1 RNA. With other small substrates, even denaturation and renaturation did not allow the detection of stable E–S complexes. This latter result suggests again that the identity of the 5′ leader sequence may be important in stabilizing the E–S interaction sufficiently to allow its detection in our system.

Nonidentity of Substrate- and Product-Binding Sites. $tRNA^{Tyr}$ inhibits cleavage of p43Tyr by M1 RNA, acting as a noncompetitive inhibitor (Figure 5). While our data are not in agreement with results from another laboratory (Reich et al., 1988; Smith et al., 1992) the noncompetitive mode of inhibition by $tRNA^{Tyr}$ is in complete agreement with our gel–shift results (see below). Our data show that M1 RNA may

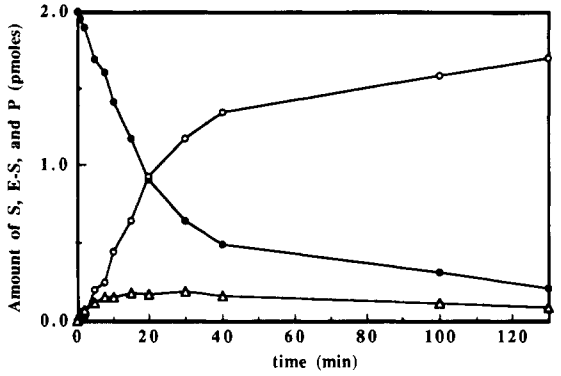

FIGURE 6: Kinetics of appearance of S, E–S, and P in the reaction catalyzed by M1 RNA. M1 RNA (10 nM) and p43Tyr (200 nM) were incubated together in 150 μL of buffer B at 37 °C. Aliquots of 10 μL each were withdrawn at the times indicated, added to 1 μL of loading dye solution, and kept on ice. The samples were analyzed on a 3% agarose gel. Efficiencies of both cleavage and binding were calculated from data obtained with a Betascope blot analyzer. (○) Amount of tRNA product formed; (●) amount of substrate remaining; (△) amount of E–S formed.

have somewhat different binding sites for the substrate and/or the product and that these sites may overlap with but may not be identical to the active site. The existence of nonidentical, possibly overlapping, binding sites that separately recognize primarily either the substrate or the product would also explain the inhibition of M1 RNA activity by substrate at a high concentration (Guerrier-Takada et al., 1989).

The Role of 2′-Hydroxyl Groups in Enzyme–Substrate Interactions. An 18-mer DNA substrate (Figure 1), corresponding in sequence to the 18-mer RNA substrate derived from p6AT1 described above, in the presence or the absence of an EGS, does not bind to M1 RNA even though annealing between the DNA substrate and the EGS is easily detectable (data not shown). If, however, certain 2′-hydroxyl groups are retained in the substrate, as in a mixed RNA/DNA substrate, 18-mer 2R-DNA, or 18-mer 3R-DNA (Figure 1), a low level of binding is detected, a further indication of the importance of specific 2′-hydroxyl groups (Perreault & Altman, 1992) in the substrate.

Steady-State (Michaelis–Menten) Kinetics of Formation of the Complex. The reaction catalyzed by M1 RNA displays Michaelis–Menten kinetics. When M1 RNA (E) is placed in the presence of p43Tyr (S), the processing reaction first requires the formation of an E–S complex. If the concentration of substrate is high enough to "saturate" the enzyme, the rate of the overall chemical process reaches a limiting value that is proportional to the concentration of the enzyme but independent of the concentration of the substrate. These standard conditions should be met and should be verifiable by the gel-shift assay if the assay does, indeed, reflect authentic interactions between M1 RNA and its substrates. The kinetics of formation of the E–S complex (Figure 6) indeed show that the concentration of E–S increases to a near constant or "steady-state" level soon after the reactants are mixed together while the formation of free P lags behind immediately after mixing. Analyses of E–S complexes have shown that they consist of enzyme (E) and substrate (S) but no product (P) (Altman et al., 1987). We cannot detect E–P complexes in our system although previous studies of M1 RNA-catalyzed reactions indicate that the release of the product is the rate-determining step in the overall reaction (Reich et al., 1988). (Those kinetic studies, with which are data are in agreement, made no distinction between cleavage of substrate and release of product from the surface of the enzyme in the final stage of the reaction. The cleavage event and product release may occur in such a manner that we cannot resolve them in time. Tallsjö & Kirsebom (1993) also report such an observation and conclude, independently, that product release is rate limiting.) As the incubation is continued, the level of E–S falls (Figure 6), suggesting that the detected complex is a productive one. This hypothesis has been verified by pulse–chase experiments (see below).

If we represent the kinetics of the M1 RNA-catalyzed reaction by

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–}S \overset{k_2}{\rightarrow} E + P \qquad (1)$$

where E–S is the only intermediate in the reaction at the steady state, the Michaelis–Menten constant $K_M = (k_{-1} + k_2)/k_1$ can be calculated from direct measurements of the concentrations of E, S, E–S, and P. The K_M, calculated in this way, for the reaction between M1 RNA and p43Tyr is approximately 1.5×10^{-7} M; this value is 5-fold higher than the value estimated from cleavage assays (3×10^{-8} M). This difference can be explained by the fact that, in the cleavage assays, the range of concentrations of the substrate was close to the concentration of substrates that inhibited M1 RNA activity and the K_M value obtained was underestimated, but when the K_M is measured in the linear range of velocity dependence on substrate concentration, a higher value of K_M is obtained (data not shown). The value of k_2 (24 h^{-1}) that we have calculated with these data is virtually identical to the value for k_{cat} calculated from the conventional Michaelis–Menten analysis (Guerrier-Takada et al., 1989). If we assume that the K_d value for the binding of pTyr to the wild-type M1 RNA is at least as low as the one observed for Δ[65] RNA (K_d = 40 nM, see below), we can calculate k_{-1} and k_1 ($K_d = k_{-1}/k_1$). Indeed, if in the equation $K_M = (k_{-1} + k_2)/k_1$ we replace K_M and k_{-1}/k_1 or K_d by their values, we determined that $k_1 = 2 \times 10^8$ M^{-1} s^{-1} and $k_{-1} = 8$ s^{-1}.

If the chemical step of the reaction is blocked, calculation of the binding constant for the substrate and the wild-type enzyme should be possible. In order to make such measurements with the wild-type enzyme, we used metal ions (Eu^{3+}, Cu^{2+}, Zn^{2+}) as inhibitors of the cleavage reaction (Kazakov & Altman, 1991) and assumed that they would not prevent formation of the E–S. Our results (data not shown) indicate that these metal ions inhibit not only the cleavage but also the binding of the substrate (p43Tyr) to M1 RNA. Therefore, we made use of a derivative of M1 RNA, which is catalytically inactive when assayed alone and has a single-point deletion at position 65 (Δ[65] RNA). Even though Δ[65] RNA is catalytically inactive when assayed alone, it does manifest activity in the presence of C5 protein. Since the RNA subunit of *E. coli* RNase P is responsible for catalysis by the enzyme, Δ[65] RNA must still retain most of the features of the wild-type active center. C5 protein may alter the binding of the substrate to the RNA. When Δ[65] RNA alone is acting as the enzyme, eq 1 becomes

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–}S \qquad (2)$$

and the dissociation constant $K_d = k_{-1}/k_1$. The K_d value obtained, namely, 40 nM, indicates tight binding of Δ[65] RNA to p43Tyr. The wild-type enzyme M1 RNA is expected to bind at least as tightly to p43Tyr. On the other hand, a variant of M1 RNA with a large deletion, Δ[156–290] RNA, which is catalytically inactive even in the presence of C5 protein, still binds to p43Tyr, but the K_d value is 100 nM

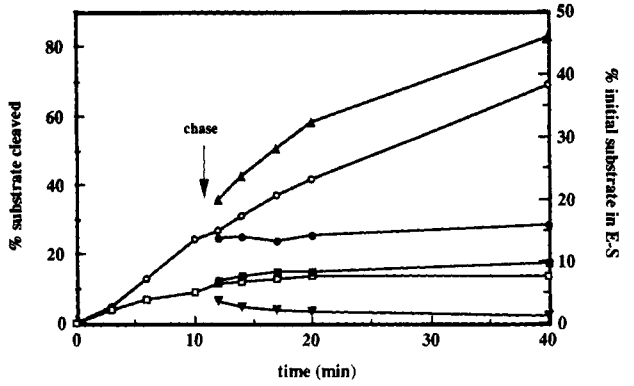

FIGURE 7: Pulse–chase of S from E–S. M1 RNA (10 nM) and p43Tyr (200 nM) were incubated together in 200 μL of buffer B for 11 min at 37 °C. Three 50-μL aliquots were withdrawn: to one was added 2 μL of H_2O; to another, 2 μL of tRNA (12.5 pmol/μL); and to the third, 2 μL of a solution of oligonucleotide complementary to nucleotides −19 to −4 ($oligo_{-19\ to\ -4}$) in the 5′ leader sequence of p43Tyr (15 pmol/μL). Incubation was performed at 37 °C for a total of 40 min, and aliquots were withdrawn at the times indicated and analyzed on a 3% agarose gel. The arrow indicates the timing of the chase. (○) % substrate cleaved in the control reaction; (□) % of initial substrate in E–S in the control reaction; (●) % substrate cleaved after addition of tRNA; (■) % of initial substrate in E–S after addition of tRNA; (▲) % substrate cleaved after addition of $oligo_{-19\ to\ -4}$; (▼) % of initial substrate in E–S after addition of $oligo_{-19\ to\ -4}$. Similar results to those shown here were also obtained using a 4-fold higher concentration of tRNA.

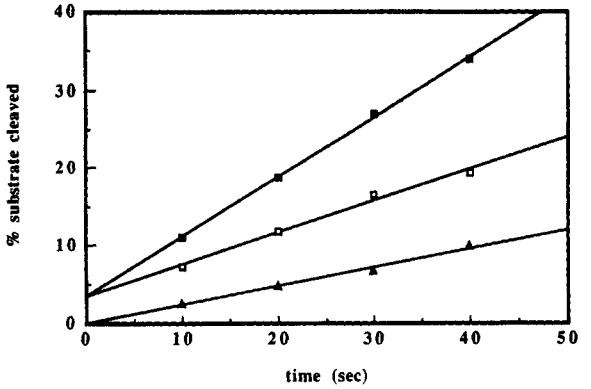

FIGURE 8: Effect of the presence of the 3′ proximal CCA sequence on the initial "burst" of product phenomenon in the reaction catalyzed by M1 RNA. For each of the reactions, M1 RNA (2.5 nM) was preincubated in buffer B for 5 min at 37 °C. The reactions were started by addition of ^{32}P-labeled p43Tyr (□), p14Tyr (■), or p43Tyr–CCAUCA (▲) as substrate (50 nM). Similar results were obtained when the p43Tyr–CCAUCA concentration was 400 nM. Aliquots were withdrawn 10, 20, 30, and 40 s after the addition of the substrate, and the products of the reaction were resolved on an 8% polyacrylamide gel that contained 7 M urea. A computer-generated linear curve fitting the experimental data was extrapolated to time zero to determine whether or not there was an initial burst in each reaction. Activity is expressed as the percentage of total substrate that was cleaved.

(Table I). This weaker binding was anticipated from results of reconstitution experiments (Guerrier-Takada & Altman, 1992) that indicated that the deleted region of M1 RNA was essential for facilitating binding of this substrate to M1 RNA prior to cleavage by the enzyme.

Kinetic Studies with Product, Various Substrates, and Fragments Thereof. The results of pulse–chase experiments, in which S is chased from the E–S complexes by tRNA or by an oligonucleotide that is complementary to the 5′ leader sequence, are in agreement with our data regarding separate binding sites for substrate and product (Figure 7). The addition of tRNA does not chase the substrate from the E–S complex but does inhibit its cleavage, a result that is compatible with a noncompetitive mechanism of inhibition. This result indicates that the binding sites for substrate and product are not identical and that cleavage cannot occur unless the binding site for the product is unoccupied. However, an oligonucleotide complementary to the 5′ leader sequence of ptRNA chases uncleaved substrate from the preformed E–S complex by competing with a binding site on the enzyme for the 5′ leader sequence of the substrate.

If the oligonucleotide complementary to the 5′ leader sequence is added to the reaction mixture at the *start* of the incubation, no formation of an E–S complex is detected, presumably because of the annealing of the 5′ leader sequence (in the substrate) to the oligonucleotide that is complementary to it. However, we find that the rate of cleavage of p43Tyr is stimulated (data not shown) in the presence of the oligonucleotide. This result indicates that strong interactions via the 5′ leader sequence in the precursor tRNA may prevent rapid release of product, and it could explain the biphasic nature of kinetics of the cleavage reaction. The oligonucleotide, by interacting with the substrate, could accelerate both the release of the 5′ leader sequence in the substrate from a strong binding site in M1 RNA and the procession of the substrate through the chemical step of the reaction. If this occurs, the cleavage of p14Tyr by M1 RNA should not exhibit the initial (pre-steady-state) "burst" of product formation (Fersht, 1985), followed by a subsequent slower rate of product accumulation, that is observed with p43Tyr. When p14Tyr is used as a substrate, we fail to detect any E–S complex, probably because of the rapid conversion of E–S to E–P (or E + P). The failure to detect E–S with p14Tyr can be rationalized on the basis of a faster conversion of E–S to E–P relative to that which occurs with p43Tyr. Our results (Figure 8) indicate, however, that cleavage of both substrates by M1 RNA is associated with the same initial burst of formation of P. Nonetheless, the steady-state rate of cleavage is about 4-fold higher in the case of p14Tyr. This difference suggests that release of the product is rate limiting for both p14Tyr and p43Tyr and that it is not the interaction of the 5′ leader sequence that prevents release of the product.

We next asked, given that a different 5′ leader sequence which does not allow the substrate to form a stable E–S complex cannot prevent the initial burst, what would be the effect of removing the 3′ CCA sequence from the substrate? Our results showed clearly that the presence of the 3′ CCA sequence in the substrate is essential for the initial burst to occur (Figure 8), while the nature of the 5′ leader sequence determines the detectability of a stable E–S complex in the gel–shift system, as indicated by the results shown in Figure 4.

Activation by Metal Ions. M1 RNA activity is stimulated by metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}; Guerrier-Takada et al., 1986; Kazakov & Altman, 1991). We examined the role of metal ions (more specifically Mg^{2+}) in the formation of E–S complexes. If we assume that there is no cleavage unless both Mg^{2+} and substrate are bound to the enzyme, two outcomes for the reaction can be envisioned: (1) no E–S complex is formed in the absence of Mg^{2+} or (2) no E–Mg^{2+} complex is formed in the absence of substrate. Binding assays performed in the absence of Mg^{2+} failed to generate stable E–S complexes. By contrast, by plotting the initial rate of the cleavage reaction versus the concentration of Mg^{2+} ions at different concentrations of substrate (data not shown) we were able to determine that the M1 RNA reaction behaves according to the first alternative cited above (Gutfreund, 1972) in which no (productive) E–S complex is found unless Mg^{2+} is present and, moreover, that substrate is not necessary for binding of

Mg^{2+} to M1 RNA by itself. Indeed, when we preincubated M1 RNA with Mg^{2+} there was no lag in the kinetics of the reaction (Guerrier-Takada et al., 1983), suggesting that the binding of Mg^{2+} facilitates a conformational change in the enzyme.

Our experiments indicate that on incubation of enzyme and substrate in buffer that contains Mg^{2+}, formation of E–S is readily detectable, independent of whether or not either enzyme or substrate has been *preincubated* separately in Mg^{2+}-containing buffer. This observation is indicative of a very high affinity of Mg^{2+} for the enzyme and/or substrate and a very rapid rate of formation of the E–S complex. The presence of Mg^{2+} is, however, absolutely required for the formation of stable E–S complexes, even though unstable complexes may form in the absence of Mg^{2+} (Smith et al., 1992).

DISCUSSION

We have described a gel–shift assay which reflects the specificity of interactions between RNA molecules that are complementary to each other and/or that have been shown to participate together in biochemical reactions *in vitro* and *in vivo* (Pyle et al., 1990; Weller & Hill, 1992). This assay has allowed us to determine binding constants for interactions between RNA molecules, as well as rate constants of different steps in enzymatic reactions that involve the RNA subunit of RNase P (M1 RNA) from *E. coli* and its substrates, and we have been able to study the conformation of M1 RNA in solution by examining the accessibility to various oligonucleotides of different regions of M1 RNA. For example, nucleotides 66–74 and 353–360 of M1 RNA are thought to form a helix that is part of a pseudoknot structure. Our data (Table I) show that the segment near the 3′ terminus of M1 RNA is relatively inaccessible to complementary oligonucleotides in solution, whereas the region from nucleotides 56–74 is relatively accessible to such oligonucleotides.

Our binding results are compatible with a three-dimensional computer-generated model of the structure of M1 RNA (E. Westhof and S. Altman, unpublished), in which nucleotides 61–74 are exposed to the solution while those around 350–360 are not. In this model, A65 is available for interactions with other nucleotides and occupies a critical position in a turn in the phosphodiester chain before the chain enters a helical section of a pseudoknot. When this nucleotide is deleted, the geometry of the surrounding region is apparently altered such that it is even more accessible to other oligonucleotides (Table I, entries 5 and 6). It might also be expected, therefore, that a deletion of A65 would severely affect the catalytic activity of M1 RNA, as it indeed does. It is not yet known why this deletion has a more deleterious effect on the cleavage of small model substrates rather than on that of p43Tyr or other ptRNAs.

In the model of the secondary structure of M1 RNA, as well as in the computer-generated model, the "top" half of the molecule forms a structural domain that is well separated from the "bottom" half (Figure 1). As anticipated, nucleotides 119–138, among all the regions tested, are the most accessible to other oligonucleotides. Nevertheless, some part of this region does seem to interact with the "bottom" domain since a region of the "bottom" domain (nucleotides 56–74) becomes more accessible to a test oligonucleotide when nucleotides 119–138 are part of a complex with a complementary oligonucleotide. Observations of this type will be fully exploited in attempts at building three-dimensional models.

Our system does not allow the detection of the formation of an E–S complex for all substrates with M1 RNA. However, when a prior heating and annealing step is carried out with the mixture of enzyme and substrate, formation of some E–S complexes that were not seen previously can be observed. Our annealing experiments show that at least some, if not all, ptRNAs undergo conformational changes as they bind to the enzyme. Tertiary structure is unfolded at an early step in the binding, and, subsequently, as inferred from the results with substrates that resemble hairpins, the acceptor stem is denatured, either at the same time as or just prior to the chemical step of the overall reaction. Our annealing experiments also suggested that the immediate product of cleavage of a ptRNA, itself a tRNA, while still bound to the enzyme, is not identical to a (refolded) tRNA that is free in solution. Furthermore, the inability of tRNA to chase substrate from E–S complexes shows that the binding sites for product and substrate on the enzyme are not precisely identical (see also below). This latter result is compatible with our results of the kinetics of inhibition of the enzymatic reaction by tRNA. It might also suggest that eq 1 be modified to include a step describing the formation of an E–P complex before the final step, but we cannot detect such a complex in our system.

Direct measurement of all the components in the enzymatic reaction (Figure 5) enabled us to calculate all the rate constants in the Michaelis–Menten equation, namely K_M, K_d, and k_{cat}. The value of K_M measured in this way is close to that estimated from classical Michaelis–Menten analysis.

All our data together indicate that an oligonucleotide complementary to the 5′ leader sequence of p43Tyr, rather than the mature tTyr, can effectively displace S from E–S complexes. Furthermore, this particular displacement mechanism triggers the progression of S in the reaction pathway to the chemical cleavage step. Subsequently, the presence of the 3′ terminal CCA sequence of P facilitates release of the product.

The unique properties of the 5′ leader sequence of p43Tyr facilitates the detection of E–S complexes in our system, independent of whether this sequence is part of p43Tyr or is grafted onto $tRNA^{Phe}$ to form a chimeric tRNA, while the 5′ leader sequences of p15Phe and other substrates do not. These data confirm what might have been an obvious prediction, that the leader sequence of a ptRNA must play some role in the E–S interactions (Altman et al., 1987; Hollingsworth & Martin, 1987). More specifically, the presence, in the 5′ leader sequence, of contiguous nucleotides complementary to a sequence in M1 RNA may significantly strengthen the binding of the substrate to the enzyme. The 5′ leader sequence interacts primarily with features determined by sequences in the 3′ half of M1 RNA, while the CCA terminus of the tRNA interacts primarily with features determined by sequences in the 5′ half of M1 RNA. Similarly, p43Tyr binds preferentially to the 3′ half of M1 RNA, while tTyr binds preferentially to the 5′ half of M1 RNA. These observations confirm directly that the binding sites for S and P are not precisely identical.

It is noteworthy that the two halves of M1 RNA form a helical region together in the "bridge" region, namely, nucleotides 75–90 and 238–246 (see Figure 1A), and that the region of the 5′ leader sequence that contains the site of cleavage by M1 RNA and the 3′ terminus of a ptRNA are also near each other in these substrates. Furthermore, cross-links with either the 5′ terminus of a tRNA or nucleotide −3 in p43Tyr are found at nucleotides 249 and 92, respectively, in M1 RNA, on either side of the bridge. We suggest, therefore, that the bridge region and its flanking sequences are the features of the enzyme that determine whether or not

a substrate will progress through the chemical step in the reaction pathway. This suggestion is in accord with the results from other studies of structure–function relationships that are associated with the cleavage reaction and our own observations that cleavage of all substrates is abolished when nucleotides 62–108 are deleted from M1 RNA.

The assays we describe here will facilitate a more detailed description of the topography of M1 RNA and of the separate steps involved in the formation of E–S and E–P complexes.

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REFERENCES

Altman, S., Baer, M., Gold, H., Guerrier-Takada, C., Kirsebom, L., Lumelsky, N., & Vioque, A. (1987) in *Molecular Biology of RNA: New Perspectives* (Inouye, M., & Dudock, B. S., Eds.) pp 3–15, Academic Press, San Diego, CA.

Fersht, A. (1985) in *Enzyme structure and mechanism*, 2nd ed., pp 98–154, W. H. Freeman and Company, New York.

Forster, A. C., & Altman, S. (1990) *Science 249,* 783–786.

Guerrier-Takada, C., & Altman, S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89,* 1266–1270.

Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., & Altman, S. (1983) *Cell 35,* 849–857.

Guerrier-Takada, C., McClain, W., & Altman, S. (1984) *Cell 38,* 219–224.

Guerrier-Takada, C., Haydock, K., Allen, L., & Altman, S. (1986) *Biochemistry 25,* 1509–1515.

Guerrier-Takada, C., Lumelsky, N., & Altman, S. (1989) *Science 246,* 1578–1584.

Gutfreund, H. (1972) in *Enzymes: Physical Principles*, pp 150–153, Wiley-Interscience, London.

Hollingsworth, M. J., & Martin, N. C. (1987) *Nucleic Acids Res. 15,* 8845–8860.

James, B., Olsen, G., Lin, J., & Pace, N. (1988) *Cell 52,* 19–26.

Kazakov, S., & Altman, S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88,* 9193–9197.

Kirsebom, L., & Altman, S. (1989) *J. Mol. Biol. 207,* 837–840.

Knap, A. K., Wesolowski, D., & Altman, S. (1990) *Biochimie 72,* 779–790.

Lumelsky, N., & Altman, S. (1988) *J. Mol. Biol. 202,* 443–454.

McClain, W., Guerrier-Takada, C., & Altman, S. (1987) *Science 238,* 527–530.

Perreault, J.-P., & Altman, S. (1992) *J. Mol. Biol. 266,* 399–409.

Pyle, A. M., McSwiggen, J. A., & Cech, T. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 8187–8191.

Reich, C., Olsen, G., Pace, B., & Pace, N. (1988) *Science 239,* 178–181.

Shiraishi, M., & Shimura, Y. (1986) *EMBO J. 5,* 3673–3679.

Smith, D., Burgin, A. B., Haas, E. S., & Pace, N. R. (1992) *J. Biol. Chem. 267,* 2429–2436.

Tallsjö, A., & Kirsebom, L. (1993) *Nucleic Acids Res. 21,* 51–57.

Vioque, A., Arnez, J., & Altman, S. (1988) *J. Mol. Biol. 202,* 835–848.

Weller, J. W., & Hill, W. E. (1992) *Biochemistry 31,* 2748–2757.