Gel Retardation Analysis of the Interaction between C5 Protein and M1 RNA in the Formation of the Ribonuclease P Holoenzyme from *Escherichia coli*†

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ABSTRACT: C5 protein binds specifically and with high affinity to M1 RNA to form the ribonuclease P holoenzyme of *Escherichia coli*. The interactions between the two subunits of the enzyme have been studied *in vitro* by a gel retardation assay. The stoichiometry of the subunits in the holoenzyme is 1:1. The dissociation constant (K_d) for the specific interactions of the subunits in the holoenzyme complex is ≤0.4 nM. C5 protein also has nonspecific affinity for M1 RNA and a variety of other RNA molecules with K_d values in the order of 10–40 nM. Scatchard analysis of binding data suggests the existence of two modes of interaction between C5 protein and M1 RNA—one high-affinity and one low-affinity mode. Regions of M1 RNA essential for formation of the specific complex with C5 protein have been defined by deletion analysis and footprinting methods. Our data show that regions of M1 RNA that interact with C5 protein are clustered into three main areas that are localized between nucleotides 41–99, 168–198, and 266–287.

Ribonuclease P (RNase P)[1] is an endoribonuclease that cleaves tRNA precursors (ptRNAs) to generate the 5′ ends of mature tRNAs, with a 3′ hydroxyl group and a 5′ phosphate group at the site of cleavage (reviewed by Altman et al. (1993)). In *Escherichia coli*, the enzyme consists of a catalytic RNA subunit (M1 RNA, 377 nucleotides) and a protein cofactor (C5, 13 800 Da). Although RNase P functions as a ribonucleoprotein *in vivo* (Kole et al., 1980) the RNA subunit alone recognizes and cleaves ptRNAs at the correct site at elevated concentrations of divalent cations *in vitro* (e.g., >20 mM Mg^{2+}; Guerrier-Takada et al., 1983). The role of M1 RNA as the catalytic component of RNase P has been well documented. By contrast, our understanding of the functions of C5 protein in the holoenzyme *in vivo* remains very limited. However, the presence of the protein *in vitro* stimulates the rate of the cleavage reaction (Guerrier-Takada & Altman, 1984) and affects the substrate specificity (Guerrier-Takada et al., 1984; McClain et al., 1987). To investigate the role of C5 protein in the RNase P holoenzyme, we have determined some of the structural aspects of the interactions between M1 RNA and the C5 protein. A gel retardation assay was developed, and a range of deletion derivatives of M1 RNA was examined for the ability of each to bind C5 protein and to participate in the cleavage of ptRNAs. This technique was used in combination with chemical footprinting experiments in order to define accurately the RNA-binding site of the protein. The gel retardation assay was also used to establish the stoichiometry for C5 protein and M1 RNA in the RNase P holoenzyme.

MATERIALS AND METHODS

Overexpression and Purification of C5 Protein. C5 protein was overexpressed and purified by the procedure of Vioque et al. (1988).

Purification of ^{35}S-Labeled C5 Protein. [^{35}S]Methionine (1 mCi, 1000 Ci/mmol; Amersham) was added to a 25-mL culture of *E. coli* BL21(DE3) that carried plasmid pARE7 (Vioque et al., 1988), at the same time as IPTG was added to a final concentration of 2 mM. The cells were grown at 37 °C in M9 medium supplemented with glucose (2 mg/mL), thiamine (1 μg/mL), $MgSO_4$ (2 mM), $CaCl_2$ (0.1 mM), and carbenicillin (100 μg/mL; Sigma, St. Louis, MO), until the optical density at 550 nm (OD_{550}) reached 0.4–0.6 prior to induction. Cells were harvested 3 h after induction with IPTG, and the radiolabeled C5 protein was purified by the procedure of Vioque et al. (1988).

The concentration of C5 protein was determined by Bradford's method (Bradford, 1976), with bovine serum albumin as the standard, or from the ratio of absorbance at 280–260 nm (Layne, 1957). Both methods gave similar values for concentrations of C5 protein.

Preparation of RNA. Large-scale transcription with T7 RNA polymerase (Promega) of M1 RNA and mutant derivatives was performed as described elsewhere (Vioque et al., 1988).

^{32}P-Labeled RNAs were prepared by transcription with T7 RNA polymerase in the presence of [α-^{32}P]GTP as described elsewhere (Guerrier-Takada and Altman, 1984).

M1 RNA was labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase after dephosphorylation with calf intestine alkaline phosphatase (Guerrier-Takada & Altman, 1984), and at the 3′ end with [5-^{32}P]pCp and T4 RNA ligase (England & Uhlenbeck, 1978).

Labeled RNAs were purified from 4% (w/v) polyacrylamide/7M urea gels by elution overnight into 300 μL of 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.01% (w/v) SDS, 1 mM EDTA, at 37 °C. Eluted RNA was precipitated directly

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[1] Abbreviations: pTyr, precursor to $tRNA^{Tyr}$ from *E. coli*; p4.5S, precursor to 4.5S RNA from *E. coli*; RNase, ribonuclease; ATP, adenosine 5′-triphosphate; pCp, cytidine 3′,5′-bisphosphate; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris[(hydroxymethyl)amino]methane; SDS, sodium dodecyl sulfate; DMS, dimethyl sulfate.

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from the elution buffer with three volumes of ethanol, washed once with 70% ethanol, and vacuum-dried.

Gel Retardation Assays. Aliquots (20 μL) containing approximately 0.1 nM of internally ^{32}P-labeled RNA were prepared in binding buffer [(20 mM K–Hepes pH 8.0, 400 mM NH_4OAc, 10 mM $Mg(OAc)_2$, 0.01% (v/v) Nonidet P-40, 5% (v/v) glycerol)] and incubated at 37 °C for 5 min. C5 protein was serially diluted to the desired concentrations (typically 0.01–100 nM) in the same buffer. The binding assay was initiated by adding 5 μL of the C5 protein to the solution of RNA to give a final volume of 25 μL. After a 10-min incubation at 37 °C, 10 μL of each reaction mixture was loaded onto a 5% (w/v) polyacrylamide gel (acrylamide/bisacrylamide, 30:1) that contained 50 mM K–Hepes pH 8.0, 1 mM $Mg(OAc)_2$, 0.01% (v/v) Nonidet P-40. Gels were run at a constant current of 35 mA for 3–4 h at 4 °C with recirculation of the running buffer [50 mM K–Hepes pH 8.0, 1 mM $Mg(OAc)_2$, 0.01% (v/v) Nonidet P-40]. After electrophoresis, gels were dried and the radioactive bands were visualized by autoradiography and quantified using a Betascope blot analyzer (Betagen Co., Waltham, MA).

Stoichiometry. ^{35}S-labeled C5 protein (93 200 cpm/pmol) and ^{32}P-labeled M1 RNA (522 000 cpm/pmol) were mixed at varying ratios (1–100 fmol RNA to 1000 fmol protein) in binding buffer and incubated for 10 min at 37 °C prior to fractionating on a native 5% (w/v) polyacrylamide gel as described above. Bands corresponding to the C5 protein-M1 RNA complex were visualized by autoradiography and excised from the gel. The gel slices were prepared for scintillation counting by crushing in glass scintillation vials and soaking overnight in 5 mL of Omnifluor (NEN) aqueous scintillant. The counting efficiency was determined by adding known amounts of [^{35}S]methionine of [^{32}P]GTP to similarly prepared blank samples.

Deletion Mutants of M1 RNA. Plasmid pJA2′ (Vioque et al., 1988) was used to generate the deletion derivatives of M1 RNA as described previously (Guerrier-Takada and Altman, 1992), with the exception of Δ79–86, Δ185–201, and Δ266–269. These latter deletion mutants were prepared by site-directed mutagenesis using the Altered Sites *in vitro* mutagenesis system (Promega). Plasmid pJA2′ (Vioque et al., 1988) was digested to generate an EcoRI-HindIII fragment that contained the bacteriophage T7 promoter followed by the gene for M1 RNA. The DNA fragment was ligated into the pALTER plasmid (Promega) that had been digested with EcoRI and HindIII to generate plasmid pALTER-M1. Deletion derivatives were prepared using the following oligodeoxynucleotides: Δ79–86, 5′-GGAAAGTCCGGGCTCCGGGTGCCAGGTAACGCC-3′; Δ185–201, 5′-GATCAGGTAAGGGTGAAAGGCGCGCGGCTGGTAACAGTCC-3′; Δ266–269, 5′-GCCAAATAGGGGTTCGGTACGGCCCGTAC-3′.

DNA from all deletion-derivative plasmids was sequenced to verify the extent of the deletions.

Assays of Catalytic Activity of M1 RNA and Deletion Derivatives. M1 RNA and its mutant derivatives were assayed with pTyr or p4.5S as substrate (Guerrier-Takada et al., 1983; Guerrier-Takada & Altman, 1992).

Chemical Footprinting. M1 RNA-C5 protein complexes were formed between 0.1 nM end-labeled M1 RNA (~50 000 cpm) and 1.0 nM C5 protein at 37 °C for 10 min in binding buffer [20 mM K–Hepes pH 8.0, 400 mM NH_4OAc, 10 mM $Mg(OAc)_2$, 0.01% (v/v) Nonidet P-40, 5% (v/v) glycerol], prior to chemical modification. For each reaction, a control was treated in parallel, with omission of the chemical reagent.

Modification with Dimethyl Sulfate. Modification with DMS was performed in 25 μL of binding buffer. The reaction was started by addition of 1 μL of 10% (v/v) DMS (Sigma). The mixture was incubated at 37 °C for 10 min and stopped by addition of 10 μL of DMS stop solution (1.0 M NaOAc, 1.0 M β-mercaptoethanol, 1 mM EDTA). After incubation on ice for 10 min the modified RNA was precipitated with three volumes of ethanol. Pelleted RNA was resuspended in 200 μL of 0.3 M NaOAc, pH 6.0, extracted once with an equal volume of phenol/chloroform, and reprecipitated with three volumes of ethanol.

The DMS-modified sites were detected by primer extension analysis as described elsewhere (Moazed et al., 1986).

RESULTS

The interaction between C5 protein and M1 RNA in the RNase P holoenzyme was investigated using a gel retardation assay. A constant, low concentration (~0.1 nM) of labeled RNA was incubated at 37 °C in binding buffer with varying concentrations of C5 protein. Aliquots of the reaction mixture were loaded onto 5% acrylamide gels after an incubation period of 10 min and the complex separated from free RNA by native gel electrophoresis. The RNA–protein complex, being larger and having a smaller net negative charge, migrates more slowly than free RNA in the gel matrix (Figure 1). The gel matrix effectively traps complexes and prevents further association or dissociation during electrophoresis. Previous studies (Fried & Crothers, 1981; Garner & Rezvin, 1981) have shown that protein–DNA complexes are apparently more stable during electrophoresis than would seem to be indicated by their kinetic stability when free in solution. This phenomenon was interpreted as a specific gel stabilization effect, termed "caging" (Fried & Crothers, 1981), whereby the gel matrix prevents diffusion of dissociated components. This conclusion is supported by our observation that the K_d values obtained for the C5 protein–M1 RNA interaction were independent of the duration of electrophoresis (data not shown).

An electrophoresis buffer of low ionic strength was used since such buffers generate less heat during the electrophoresis and increase the speed of migration by increasing the fraction of the current carried by the macromolecules.

Stoichiometry. The stoichiometry of protein and RNA subunits in the RNase P holoenzyme was determined by performing gel retardation experiments with ^{35}S-labeled C5 protein and ^{32}P-labeled M1 RNA. The gel retardation method separates specifically interacting C5 protein–M1 RNA complexes from free M1 RNA and protein. This method has been used to determine the stoichiometry of repressor–DNA interactions (Hendrickson & Schleif, 1985) and cAMP–cAMP receptor protein binding (Fried & Crothers, 1983; Garner & Rezvin, 1982). The labeled C5 protein and M1 RNA of known specific activity were mixed at different molar ratios (1000:1 to 10:1) in binding buffer and fractionated on native gels. Radioactive bands corresponding to complexes were visualized by autoradiography, excised, and radioactivity was quantitated in a liquid scintillation counter. The relative amounts of protein and RNA in each band were determined from the known specific activity of each. The values given in Table 1 correspond to a stoichiometry of one protein monomer bound to one M1 RNA. These data are in agreement with the unit stoichiometry calculated by Vioque et al. (1988), and also with the fact that only one specific complex is observed in the gel retardation assay over a range of concentrations of C5 protein or M1 RNA.

Specificity of the Interaction between C5 Protein and M1 RNA. A binding buffer of relatively high ionic strength was

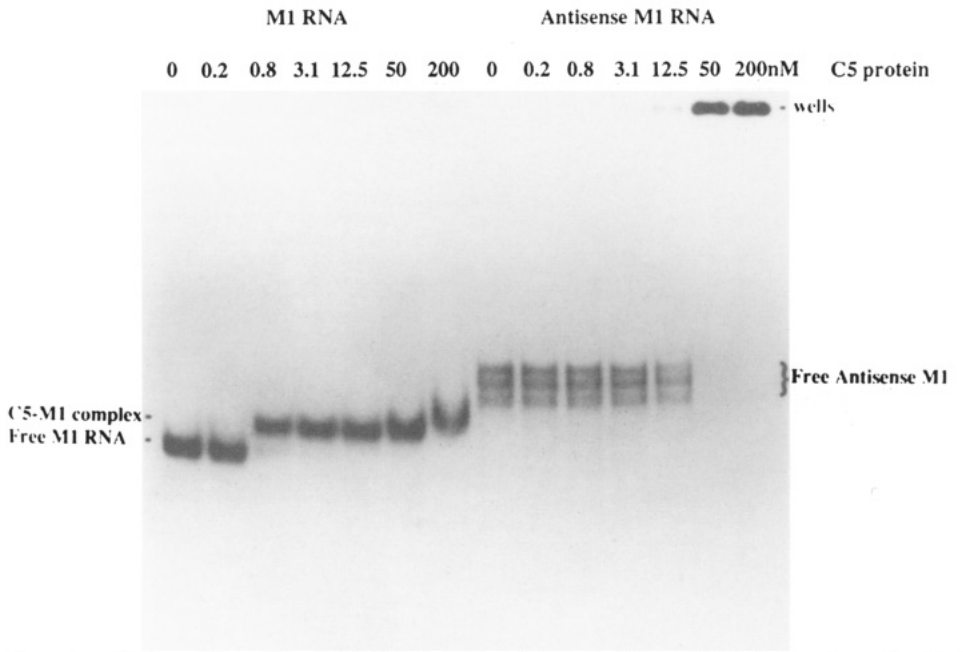

FIGURE 1: Analysis of complexes between C5 protein and M1 RNA or C5 protein and antisense M1 RNA by polyacrylamide gel electrophoresis. The more rapidly moving band on the autoradiogram corresponds to free RNA and the slower one to the RNase P holoenzyme complex. Several bands are observed with antisense M1 RNA due to the formation of multiple conformations in the native gel. At concentrations of C5 protein ≥40 nM antisense M1 RNA is observed in the wells.

Table 1: A Monomer of C5 Protein Binds M1 RNA in the Formation of the RNase P Holoenzyme

input M1 (fmol)	^{32}P cpm in complex band[a]	M1 in M1–C5 complex band (fmol)	^{35}S cpm in complex band[a]	C5 in M1–C5 complex band (fmol)	ratio (fmol of C5/fmol of M1 in RNase P)
1	501	0.96	91	0.97	1.01
10	4 490	8.6	970	10.4	1.21
50	25 200	48.3	4 620	49.5	1.03
100	49 900	95.6	9 300	99.7	1.04

[a] The specific activities of [^{32}P]M1 RNA and [^{35}S]C5 protein were 522 000 cpm/pmol and 93 200 cpm/pmol, respectively.

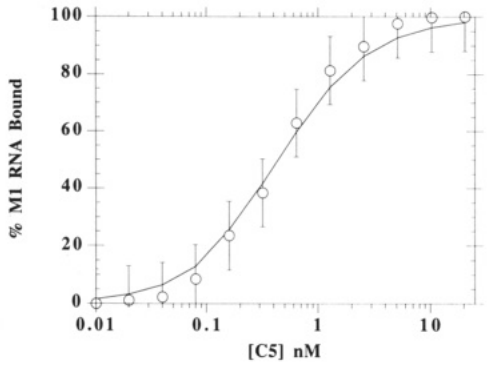

FIGURE 2: C5 protein excess binding curve for M1 RNA. Binding reactions were incubated for 10 min at 37 °C in binding buffer, before loading onto native polyacrylamide gels. The dissociation binding (K_d) was determined by fitting a binding curve (with K_d = 0.4 nM in this case) to the empirical data.

used to ensure specific interactions between C5 protein and the target RNA. Vioque et al. (1988) showed that at concentrations of monovalent salts below 400 mM, the nonspecific binding of C5 protein to various RNAs unrelated to M1 RNA is significant. As shown in Figures 1 and 2, the extent of formation of the M1 RNA–C5 protein complex was detectable at 0.08 nM C5 protein, had a midpoint at 0.4 nM, and reached a plateau above 5.0 nM, with 100% of the RNA bound in one specific type of complex in the gel. The dissociation constant (K_d) for formation of the C5 protein–M1 RNA complex was calculated from the real binding curve to be 0.4 nM. At concentrations of C5 protein above 40 nM a nonspecific aggregate of protein and RNA was observed in the wells of the gel. [At high concentrations, C5 protein is insoluble in the absence of urea (Vioque et al., 1988).] An estimate of K_d using data from filter-binding experiments (Vioque et al., 1988) gave a value (0.4 nM) close to that obtained with the gel retardation assay described here. Gel retardation, appears however, to be a more accurate method than the filter-binding assay for determining binding constants since 100% of the input RNA is actually observed in the complex (Figures 1 and 2).

Throughout this and the accompanying paper the concentration of C5 protein given is the total concentration used in the experiments reported here. It has been found, however, that in some preparations of C5 protein only 25–50% of the protein is active in the enzymatic assay (Vioque et al., 1988). Accordingly, it is possible that the actual concentration of (unlabeled) protein bound to M1 RNA in the gel shift assay is lower than the nominal value (if enzymatically inactive protein is also incapable of binding to M1 RNA) and, therefore, all numbers cited for measured binding constants are maxima.

The interaction between C5 protein and M1 RNA was investigated by Scatchard analysis. By using a concentration of C5 protein near the K_d (0.64 nM), the concentration of labeled M1 RNA was varied over a range that spanned the K_d (0.1–100 nM). These data were analyzed on a Scatchard plot (r/C_f versus r; where r = [RNA in complex]/[total protein], and C_f = [free RNA]), as shown in Figure 3. The nonideal binding behavior, indicated by the concave curve, complicates determination of stoichiometry from the plot. However, the ordinate gives a ratio of 1 to 1.06 for the subunits in the complex. Accordingly, an explanation for such nonideal

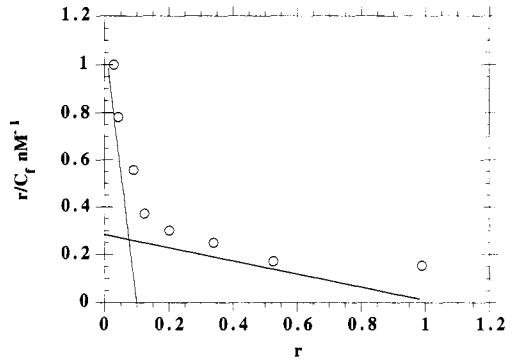

FIGURE 3: Scatchard plot for binding of C5 protein to M1 RNA, where r = [RNA in complex]/[total protein] and C_f = [free RNA] the lines are drawn to give the best fit to the data using the method of Zierler (1989). Complexes were formed between a constant amount of C5 protein (0.64 nM) and concentrations of M1 RNA from 0.1 to 100 nM. K_d values were determined from the reciprocal of the slope of the line at low concentrations of M1 (K_d = 0.1 nM) and at high concentrations of M1 ($K_d \approx 5$ nM).

Table 2: Dissociation Constants (K_d) for C5 Protein Binding to a Variety of RNAs

RNA	K_d (nM)
M1 RNA (*E. coli*)	0.4
P-RNA (*B. subtilis*)	1.2
H1 RNA (*Homo sapiens*)	>40
pTyr	>40
p4.5S	>40
M1 + pTyr	0.4
M1 + p4.5S	0.4
antisense M1 RNA	>40

binding behavior is that there are two (or more) modes of binding of C5 protein on M1 RNA with different fixed affinities. Analysis of Figure 3 suggests that two modes of binding exist—one high-affinity mode (K_d = 0.1 nM) and the other low-affinity mode ($K_d \approx 5$ nM). The low-affinity mode of binding may reflect the formation of aggregates at concentrations of active C5 protein above 40 nM. The higher affinity (K_d = 0.1 nM) is in reasonable agreement with the K_d determined from the excess C5 protein binding curve (K_d = 0.4 nM; Figure 2).

Table 2 gives K_d values for the interaction of C5 protein with various species of RNA. When a binding assay was performed with antisense M1 RNA, no specific complex was observed in the gel (Figure 1). At high concentrations of C5 protein (≥40 nM) the RNA was observed in the wells. This result may reflect a nonspecific interaction between the basic C5 protein and the negatively charged RNA which results in aggregates that are incapable of entering the gel matrix. This nonspecific binding was also observed with M1 RNA at concentrations of C5 protein above 20 nM. C5 protein interacts specifically with P-RNA—the RNA moiety from RNase P of *Bacillus subtilis*—with a K_d 3-fold higher than that for M1 RNA, but it has a low nonspecific affinity for H1 RNA (the RNA subunit from human RNase P). No specific complexes were formed between C5 protein and substrates of RNase P, namely, pTyr and p4.5S. In addition, these two substrates had no effect on the affinity of C5 protein for M1 RNA.

The affinities of C5 protein for P-RNA and of P-protein (the protein subunit of RNase P from *B. subtilis*) for M1 RNA were measured (Table 3). The dissociation constants determined by the gel retardation assay indicated that each protein has a roughly similar affinity (0.4 nM) for its respective RNA, but a 3–4-fold reduced affinity for the corresponding

Table 3: Dissociation Constants (K_d) for C5 or P-Protein Binding to M1 or P-RNA[a]

RNA	K_d (nM)	
	C5 protein	P protein[b]
M1	0.4	1.6
P-RNA	1.2	0.4

[a] P protein and P-RNA are the protein and RNA moieties from *B. subtilis* RNase P. [b] P protein and a clone harboring the gene for P-RNA downstream from a T7 RNA polymerase promoter were gifts from B. Pace and N. Pace (Indiana University).

Table 4: Dissociation Constants (K_d) for Binding of C5 Protein and Catalytic Activities of Various Deletion Derivatives of M1 RNA

RNA	K_d (nM)	% activity[a] (RNA alone)		% activity[a] (RNA + C5)	
		pTyr	p4.5S	pTyr	p4.5S
M1	0.4	100[b]	100[c]	100[b]	100[c]
Δ92	0.4	0.15[b]	100[c]	7.7[b]	22[c]
Δ65	4.0	0[b]	0	1.4[b]	0
Δ79–86	1.6	0	0	0	0
Δ273–281	1.6	92[c]	118[c]	97	1.6[c]
Δ266–269	0.4	85	109	102	87
Δ185–201	10–40	0.003	75	0.000 07	9
Δ156–205	>40	0[c]	55[c]	0[c]	6[c]
Δ93–104	2.4	0[c]	0[c]	0[c]	6
Δ94–204	>40	0[c]	0[c]	0[c]	9[c]
Δ62–108	>40	0[d]	0	0	0
Δ1–163	>40	0[d]	0	0	0
Δ169–377	>40	0[d]	0	0	0
Δ1–54	>40	0[d]	0	0	0
Δ1–54, 273–377	>40	0[d]	0	0	0

[a] % activity is given relative to the ratio of k_{cat}/K_m for wild-type M1 RNA or the RNase P holoenzyme. The values of k_{cat}/K_m for the cleavage of pTyr or p4.5S by M1 RNA alone are 13 and 0.11 min^{-1} μM^{-1}, respectively. The values of k_{cat}/K_m for the cleavage of pTyr or p4.5S by the RNase P holoenzyme are 880 and 400 min^{-1} μM^{-1}, respectively. [b] Guerrier-Takada et al. (1989). [c] Unpublished (C. Guerrier-Takada and S.A.). [d] Guerrier-Takada and Altman (1992). The values of the kinetic constants for these mutants are all zero.

RNA from the other organism. It has been shown previously that C5 and P proteins can be mixed with the heterologous P and M1 RNAs to form functional hybrid holoenzymes (Guerrier-Takada et al., 1983).

Affinity of C5 Protein for Deletion Derivatives of M1 RNA. The dissociation constants for binding of C5 protein to various deletion derivatives of M1 RNA were determined (Table 4). The deletion derivatives fell into three main classes: (i) RNAs that do not bind C5 protein and are catalytically inactive; (ii) RNAs that bind C5 protein but have a reduced catalytic activity; and (iii) RNAs that are essentially wild type with respect to binding of C5 protein and catalytic activity. It was demonstrated previously that the rate of cleavage of tRNA precursor substrates by RNase P is enhanced 20–50-fold by the presence of the C5 protein, even though M1 RNA is solely responsible for binding of the substrate (Guerrier-Takada et al., 1983). In the case of Δ92 and Δ266–269 where binding to C5 protein was "wild type", stimulation of catalytic activity in the presence of C5 protein was also observed. Deletion of A65 completely abolishes catalytic activity in the absence of C5 protein and, in addition, this derivative had a 10-fold lower affinity for C5 protein. Deletion of residues 79–86 or 273–281, which have been proposed to base pair in a pseudoknot interaction (Haas et al., 1991), resulted in derivatives with a 3–4-fold lower affinity for C5 protein. However, Δ79–86 was catalytically inactive, in contrast to Δ273–281 which was essentially wild type in terms of activity. The loop region between nucleotides 92–204 appears to be critical for stim-

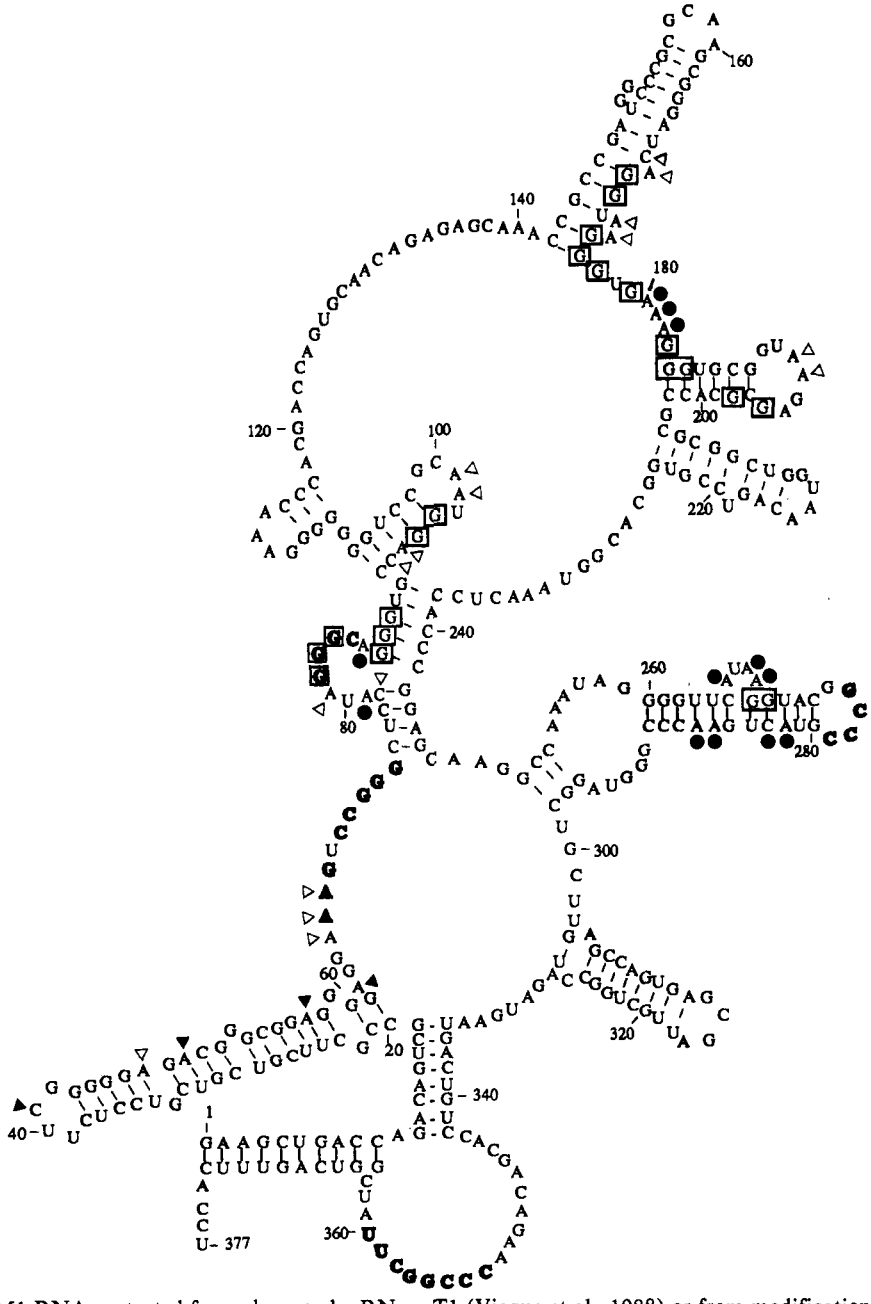

FIGURE 4: Regions of M1 RNA protected from cleavage by RNase T1 (Vioque et al., 1988) or from modification by DMS in the presence of C5 protein. Boxed regions show decreased sensitivity by RNase T1, and residues with symbols next to them indicate decreased sensitivity to modification by DMS: ▲, strongly protected; △, moderately protected; ●, weakly protected. Residues 82–85/276–279 and 66–74/353–360 (bold type) has been reported to base pair to form pseudoknot structures (Haas et al., 1991).

ulating the rate of cleavage of ptRNA. Deletion of large portions of this region completely abolished catalytic activity (with pTyr) and severely affected recognition and binding of C5 protein. It has been noted previously that deletion mutants with deletions in this region of M1 RNA, while completely inactive with pTyr as a substrate, are still capable of specifically cleaving p4.5S. This result suggests the possibility of separate or overlapping binding sites for ptRNA precursors and other non-tRNA substrates such as p4.5S RNA. In general, deletion of large regions of M1 RNA has a deleterious effect both on the catalytic activity and on recognition by C5 protein.

Deletion of regions protected by C5 protein in footprinting experiments (Figure 4) i.e., Δ79–86, Δ93–104, Δ185–201, and Δ266–269, had a wide range of effects. The affinity of C5 protein for Δ79–86, Δ93–104, or Δ185–201 was between 4- and 100-fold lower than for M1 RNA. However, deletion of residues 266–269 had no effect on either binding of C5 protein or the catalytic activity of the RNA.

Chemical Footprinting—Binding of C5 Protein to M1 RNA. To delineate the M1 RNA binding site recognized by C5 protein, a chemical footprinting approach was employed. After formation of the RNase P holoenzyme in binding buffer, the end-labeled M1 RNA in the complex was probed with the chemical reagent dimethyl sulfate (DMS, which methylates the N1 of adenine, the N3 of cytidine, and N7 of purines). RNA modified at a particular residue was then analyzed by primer extension (data not shown). Three distinct regions of M1 RNA (on the secondary-structure model) are protected from modification by this reagent in the presence of C5 protein (Figure 4): residues 41–99, 168–198, and 266–271/282–287. These data are consistent with those of Vioque et al. (1988), who noted that C5 protein protected similar regions of M1

Table 5: Dissociation Constants (K_d) for C5 Protein Binding to M1 RNA in the Presence of Oligodeoxynucleotides Complementary to Regions of M1

complementary region on M1	concentration of oligonucleotide[a] (nM)	K_d (C5 + M1) (nM)	M1–oligo complex[b]	M1–oligo–C5 complex[b]
–	0	0.4		
56–74	700	>40	+	–
82–100	2000	1.6	+	–
119–138	250	0.5	+	+
152–179	1000	>40	+	–

[a] Oligonucleotides were used at concentrations 5-fold higher than their respective K_ds for M1 RNA to ensure close to 100% complex formation. [b] The two right-hand columns denote the observance of the indicated complexes in the gel.

RNA against digestion with RNase T1. However, protection from RNase T1 by digestion of residues in the stem–loop from 19 to 61 was only observed under conditions of low ionic strength or at high concentrations of C5 protein, suggesting that this region was available for nonspecific binding. It is important to note that protected residues could result from either close contact with C5 protein or from a conformational change in M1 RNA induced by binding of the protein.

Antisense Inhibition of the Binding of C5 Protein to M1 RNA. As a complementary approach to footprinting, inhibition of the binding of C5 protein to M1 RNA was investigated using antisense oligodeoxynucleotides (Table 5). After hybridization of specific oligonucleotide to a low concentration (~0.1 nM) of radiolabeled M1 RNA, the capacity of the resulting complex to bind C5 protein in the gel retardation assay was investigated. Oligonucleotides were used at 5-fold higher concentrations than their K_d values for M1 RNA to ensure close to 100% formation of M1–oligonucleotide complex. The dissociation constants were determined under identical conditions to those used for the analysis of the formation of the RNase P holoenzyme. As shown in Table 5, oligonucleotides complementary to regions of M1 RNA protected in footprinting experiments by C5 protein inhibited formation of the complex. This result is evident from the 4–100-fold increase in K_d and the absence of M1–oligonucleotide–C5 complexes in the gel (data not shown). Oligonucleotides complementary to other regions of M1 RNA had no effect on the subsequent formation of the RNase P holoenzyme. The regions of M1 RNA protected in footprinting experiments by C5 protein were the most accessible to binding of oligonucleotide (in the absence of C5), an indication that these nucleotides are positioned near the surface of the RNA molecule.

DISCUSSION

C5 protein plays an essential albeit noncatalytic role in the function of the RNase P holoenzyme *in vivo* (Kole et al., 1980; Guerrier-Takada et al., 1983). The catalytic activity and recognition of substrates by RNase P are governed mainly by the 377-nucleotide RNA subunit of the enzyme (M1 RNA; *E. coli*). In the complex with C5 protein, however, the rate of catalysis by M1 RNA is increased 20–50-fold (Guerrier-Takada et al., 1983).

We have used a gel retardation assay to study the interaction of C5 protein with its binding site(s) on M1 RNA. This technique is perhaps the simplest and most widely used technique for investigating interactions between proteins and nucleic acids. As has been noted previously (Vioque et al., 1988), C5 protein exhibits a nonspecific affinity ($K_d \geq 40$ nM) for RNAs other than M1 RNA but high specific affinity for M1 RNA ($K_d = 0.4$ nM) and closely related homologues (e.g., P-RNA from *B. subtilis*; $K_d = 1.2$ nM), as demonstrated by the gel assay. It is, perhaps, not surprising that C5 and P-proteins have high specific affinity for their respective heterologous RNA subunits since it has been demonstrated previously that catalytically active hybrids can be formed with these two enzymes. C5 protein does not, however, form any specific complex with H1 RNA (from human RNase P), despite the fact that this RNA can be folded into a secondary structure similar to that of M1 RNA (Altman et al., 1993). The human RNase P holoenzyme is known to have a higher proportion of its mass provided by the protein subunit(s) (as much as 50%, compared with 10% for prokaryotic RNase P; Bartkiewicz et al., 1989), and therefore, it might be expected that the mechanism of complex formation is very different. To date, very little is known about the protein moiety of eukaryotic RNase P, although it has been proposed (Altman et al., 1993) that the protein subunit(s) play a more fundamental role in recognition of the substrate and/or catalysis than in *E. coli* since the RNA subunit has not been shown to be catalytically active by itself *in vitro* (Bartkiewicz et al., 1989).

The binding data generated with C5 protein and deletion derivatives of M1 RNA suggest that only formation of a specific C5 protein–M1 RNA complex leads to stimulation of catalytic activity with pTyr as a substrate. Deletion derivatives of M1 RNA that did not form specific complexes that could be visualized in the gel matrix were either catalytically inactive or did not show the characteristic 20–50-fold enhancement of activity upon addition of C5 protein. The exception to this rule was Δ79–86, which had measurable affinity for C5 protein that was only 4-fold lower than that of wild-type M1 RNA, but it was completely inactive with either pTyr or p4.5S as substrate. This result suggests that, although formation of a specific C5 protein–M1 RNA complex still occurs with this deletion, some residues critical for the catalytic step have been removed. It was shown previously (Guerrier-Takada et al., 1989) that a UV-induced crosslink can be formed between C92 in M1 RNA and C-3 in the leader sequence of the precursor substrate. Furthermore, it has been proposed that a Mg^{2+} ion bound in the vicinity of the internal bulge formed by nucleotides 254–259 and 291–295 participates in the chemical step of the cleavage reaction (Kazakov & Altman, 1991). These data point to the conclusion that the catalytic center of RNase P is located in or around the central "bridge" region of M1 RNA that includes residues 79–86.

Some deletion derivatives of M1 RNA (nucleotides 92–204), while completely inactive with pTyr as a substrate, were still capable of catalyzing the specific cleavage of p4.5S. Therefore, certain nucleotides in M1 RNA that are responsible for recognition and binding of the substrate are different for pTyr and p4.5S. Presumably, some critical contact necessary for initial promotion or maintenance of the ptRNA–M1 RNA but not the p4.5S–M1 RNA complex has been removed in these deletion derivatives.

The data presented herein suggest that the site for recognition of C5 protein on M1 RNA is complicated. In contrast with simpler systems, in which a protein recognizes a short stem–loop RNA structure (e.g., R17 coat protein, Carey et al., 1983; HIV Tat protein, Weeks et al., 1990; T4 gp32, von Hippel et al., 1982), the formation of the RNase P holoenzyme is analogous to interactions between ribosomal protein and RNA. In these cases, the RNA-binding proteins generally bind to noncontiguous regions of their RNA

molecules, thereby complicating the analysis.

Some details about the site of recognition on the RNA for C5 protein can be deduced from our footprinting results. The protein apparently forms several contacts at sites which, though widely dispersed in the secondary structure of M1 RNA, must be held in a particular orientation by the tertiary structure of the RNA. Molecular modeling of M1 RNA (E. Westhof and S. Altman, unpublished) suggests that M1 RNA can be folded in such a way as to bring the protected sites in close enough proximity that they can make contact with a protein of the size of C5. In addition, this model positions the substrate-binding site such that the substrate makes no contact with the protein, in agreement with the binding data presented here, which show that the affinity of C5 protein for M1 RNA does not change in the presence of the substrate. It is noteworthy (Vioque et al., 1988) that there is a considerable degree of sequence and structural similarity in the nucleotide sequences between positions 81–101 and 174–194 in M1 RNA, namely, in regions protected by C5 protein:

$$5'\text{-}^{81}\underline{\text{AGGGCAGGGUGCCAGGUAACG}}^{101}\text{-}3'$$

$$5'\text{-}^{174}\underline{\text{AGGGUGAAAGGGUGCGGUAAG}}^{194}\text{-}3'$$

In particular, the sequences in the loop regions may be of significance for recognition by the protein. It is becoming increasingly clear that stem–loop structures and single-stranded bulges are important sites for specific RNA–protein interactions.

In the following paper (Talbot & Altman, 1994), we report the kinetic, thermodynamic, and solution properties of the interaction between C5 protein and M1 RNA.

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