A chemical interference study on the interaction of ribosomal protein L11 from *Escherichia coli* with RNA molecules containing its binding site from 23S rRNA

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ABSTRACT

The interaction between ribosomal protein L11 from *Escherichia coli* and *in vitro* synthesized RNA containing its binding site from 23S rRNA was characterized by identifying nucleotides that interfered with complex formation when chemically modified by diethylpyrocarbonate or hydrazine. Chemically modified RNA was incubated with L11 under conditions appropriate for specific binding of L11 and the resulting protein-RNA complex was separated from unbound RNA on Mg²⁺-containing polyacrylamide gels. The ability to isolate L11 complexes on such gels was affected by the extent of modification by either reagent. Protein-bound and free RNAs were recovered and treated with aniline to identify their content of modified bases. Exclusion of RNA containing chemically altered bases from L11-associated material occurred for 29 modified nucleotides, located throughout the region corresponding to residues 1055–1105 in 23S rRNA. Ten bases within this region did not reproducibly inhibit binding when modified. Multiple bands of RNA were consistently observed on the nondenaturing gels, suggesting that significant intermolecular RNA-RNA interactions had occurred.

INTRODUCTION

Interactions between ribosomal proteins and ribosomal RNA (rRNA) provide the basis for assembly of functional ribosomes; at the same time, they provide an abundant set of model systems in which one can identify structural features of RNA that mediate specific complex formation with proteins (1,2) and for which one can characterize thermodynamic parameters (3). One such interaction, that of ribosomal protein L11 from *Escherichia coli* with its binding site on 23S rRNA, is particularly interesting because it is associated with several biologically important activities including antibiotic (thiostrepton) binding (4,5), GTPase activity (reviewed in 6), possible functional long-range interactions between domains II and VI (7), stringent response

(8,9), site of attachment by elongation factor EF-G (1,10), and modulation of release factor binding (11).

The binding site for L11 has been well characterized in terms of its location (12), residues protected from chemical and enzymatic probes (13), minimum size (14) and thermodynamic parameters (14). It is a highly conserved region of 23S rRNA (15) and numerous heterologous interactions between evolutionarily distant counterparts to *E. coli* L11 and its binding site have been reported (4,15–17). Possible tertiary interactions (18) and effects of site-directed mutations throughout the L11 binding site on equilibrium constants for the L11 and thiostrepton interactions (19) have also been reported recently.

In order to identify features of the L11 binding site that are required intact for specific interaction with L11, we have subjected *in vitro* synthesized transcripts that contain the L11 binding site to chemical modification prior to complex formation. Both diethylpyrocarbonate (DEP) and aqueous hydrazine were used to partially modify the RNA under denaturing conditions such that an average of one modified base, randomly located throughout the sequence, was present in each molecule (20). L11-bound material was then separated from free RNA on Mg²⁺-containing gels and its content of modified bases was determined by analysis of aniline-induced cleavage products. Chemically modified bases that interfered with L11 binding, or complex stability on the native gels, were absent from, or were present in reduced amounts in, the L11-bound RNA.

We have used similar damage-selection experiments to characterize S8/16S rRNA (21), L1/L1-specific RNA (P.B.F.Cahill, D.L.Thurlow and R.A.Zimmermann, manuscript in preparation), 5S rRNA/L18 (D.L.Thurlow and R.A.Zimmermann, manuscript in preparation), nucleotidyl-transferase/tRNA (22), and RNase P RNA/tRNA (23) interactions. The results of such experiments, including the present study, suggest that stable ribosomal protein/rRNA interactions are mediated by folding patterns of the RNA that are easily disrupted by altering any one of many nucleotide bases throughout the protein binding site. In contrast, chemical modification of only a few discrete sites along a tRNA molecule interfere with interactions between enzymes and tRNAs.

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MATERIALS AND METHODS

All buffers were prepared using reagent grade chemicals and deionized distilled water (Barnstead NANOpureII). Solutions were autoclaved or filter sterilized and were stored at 4°C. DEP was purchased from Sigma (St. Louis, MO). Aniline and anhydrous hydrazine were prepared by distillation as described by Perrin *et al.* (24). [α - 32 P]ATP (>3000 Ci/mmol) and [γ - 32 P]ATP (>7000 Ci/mmol) were obtained from ICN Radiochemicals (Irvine, CA). Sequenase DNA sequencing kit, bacteriophage T7 RNA polymerase, phage T4 polynucleotide kinase, RNase-free DNase I, calf intestinal phosphatase, and restriction endonucleases were purchased from US Biochemical (Cleveland, OH). Bacteriophage TR4 RNA ligase was supplied by New England Biolabs (Beverly, MA) and RNaguard ribonuclease inhibitor by Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ).

Protein L11 was generously supplied by Dr. Robert A. Zimmermann (Univ. of Massachusetts, Amherst, MA). It was stored in buffered 6M urea (25) and dialyzed into 50 mM Hepes-OH (pH 7.6), 20 mM MgCl₂, 1 mM dithiothreitol (DTT), 300 mM KCl (binding buffer) immediately before incubation with RNA.

Plasmid pPS2 was constructed by inserting a 7R4-base HaeIII restriction fragment from the cloned *rmB* operon of *E. coli*, corresponding to bases 1043–1116 in domain II of 23S rRNA, into the T7 RNA polymerase transcription vector pTZ19R (US Biochemical). The construct was verified by DNA sequencing.

The insert was situated such that transcription using T7 RNA polymerase gave a full length transcript containing 42 extra bases at the 5' end and 18 extra bases at the 3' end, corresponding to segments of the multiple cloning site in pTZ19R.

Prior to *in vitro* transcription, plasmid pPS2 was linearized with EcoRI, extracted with phenol/chloroform, precipitated with ethanol and resuspended in water. Transcription of 5 μ g of

linearized DNA was carried out for 1–2 h at 37°C using phage T7 RNA polymerase (100 U) in 100 μ L of 40 mM Tris-HCl (pH 7.6), 8 mM MgCl₂, 10 mM NaCl, 5 mM DTT, 2 mM spermidine, 1 mM ATP, 1 mM GTP, 1 mM CTP, 1 mM UTP, 0.5 mg/mL bovine serum albumin, and 1 U/ μ L RNaguard ribonuclease inhibitor. To prepare uniformly 32 P-labeled transcript, the concentration of ATP was reduced to 0.6 mM and 50 μ Ci of [α - 32 P]ATP was included. The DNA template was removed by incubation with 1 U of RNase-free DNase I per μ g of DNA for 15 min at 37°C. A small aliquot was analyzed on a 1.2% agarose gel in 100 mM Tris-borate (pH 8.3), 2.5 mM Na₂EDTA (TBE) in the presence of ethidium bromide and the yield of RNA transcript was estimated according to the intensity of fluorescence. Following extraction with TE-saturated phenol, the RNA was precipitated with ethanol, resuspended in 10 mM Tris-HCl (pH 7.6), 1 mM Na₂EDTA (TE), and divided into two aliquots. One aliquot was dephosphorylated using 0.01 U of calf intestinal phosphatase per pmole of RNA (26).

The full-length transcripts were purified on a 6% polyacrylamide sequencing gel (acrylamide:N,N'-methylene bisacrylamide, 19:1) containing 8 M urea/TBE and visualized by UV shadowing. Following elution from the gel slice by soaking overnight in 0.5 M ammonium acetate, 0.1 mM EDTA, the RNA was precipitated with ethanol, resuspended in 0.3 M sodium acetate, reprecipitated, rinsed with ethanol, dried, and resuspended in TE buffer. The recovery of purified transcript was estimated by visualizing an aliquot on a 1.2% agarose gel in the presence of ethidium bromide.

The dephosphorylated RNA was labeled at its 5' end using [γ - 32 P]ATP and polynucleotide kinase (26); untreated purified transcript was labeled at its 3' end using [5'- 32 P]pCp and RNA

Figure 1. Autoradiogram showing L11-specific complexes with DEP-treated RNA, isolated on Mg²⁺-containing polyacrylamide gels. Runoff transcripts of plasmid pPS2 were labeled with 32 P; lanes 1–2, randomly labeled using [α - 32 P]ATP; lanes 3–4, 3' labeled; and lane 5, 5' labeled. The RNA was unmodified (lanes 1–2) or treated with DEP (lanes 3–5), and then incubated in the absence (lanes 1 and 3) or presence (lanes 2, 4 and 5) of L11. The upper and lower major bands observed in the absence of L11 are labeled U and L respectively. The position of L11-specific bands are indicated with arrows.

Figure 2. Autoradiogram showing L11-specific complexes with hydrazine-treated RNA, isolated on Mg²⁺-containing polyacrylamide gels. Runoff transcripts of plasmid pPS2 were labeled with 32 P; lanes 1–4, randomly labeled using [α - 32 P]ATP; lanes 5–6, 3' labeled; and lane 7, 5' labeled. The RNA was unmodified (lane 1) or treated with 50% hydrazine for 5 min (lane 2), 25% hydrazine for 5 min (lane 3), or 50% hydrazine for 2.5 min (lane 4). It was then incubated in the absence (lane 5) or presence (lanes 1–4, 6, and 7) of L11. Samples not containing L11 were made 4% in glycerol just prior to loading. The position of L11-specific bands are indicated with arrows.

ligase (27,28). End-labeled RNAs were purified on a 6% polyacrylamide sequencing gel, run at 30 mA constant current until the xylene cyanole tracking dye had migrated 12 cm ($T \approx 45^\circ\text{C}$). Full-length products were visualized by autoradiography and recovered from the gel as described above.

End-labeled RNAs, together with 2 μg of unlabeled transcript, were treated with DEP in a manner similar to that described by Peattie (20), except that the amount of DEP was reduced to 1 μL of freshly prepared 50% DEP in ethanol and the time was shortened to 1 min. Treatment with aqueous hydrazine was also carried out following the procedure of Peattie (20), except that the concentration of hydrazine was reduced to 25% and the time on ice was shortened to 5 min.

Modified and untreated control RNA (normally 6 μ M) was renatured at 42°C for 15 min in 5 μ L of binding buffer, followed by slow cooling to room temperature and 10 min on ice. Protein L11 (10 μ L) was heated at 42°C for 30 min, slow cooled to room

temperature, and chilled on ice for 10 min (29). Complex formation was carried out on ice for 1.5 h at a protein:RNA molar ratio of 2:1. The protein-RNA mixtures were then loaded directly onto a 6% polyacrylamide gel (250 mm×180 mm×1 mm) in 50 mM Tris-borate (pH 7.6), 10 mM magnesium acetate, which was run in the cold (4°C) at 15 mA constant current until the xylene cyanole tracking dye (in a separate lane) had migrated 10 cm. Bands containing protein-RNA complexes and unbound RNA were located by autoradiography, and the RNA was recovered from the gel slice as described above, except the elution buffer also contained 0.1% (w/v) SDS and 10 µg of carrier tRNA.

Following precipitating with ethanol, resuspending in 0.3 M sodium acetate, reprecipitating, rinsing with ethanol, and drying, the final RNA pellets were resuspended in 20 μ L of 1 M aniline acetate and incubated in the dark at 65°C as described by Peattie (20); the time of incubation was shortened to 5 min. After two rounds of washing with water and drying, the samples were

Figure 3. Autoradiogram of aniline-induced fragments from free and L11-bound RNA, previously treated with DEP. RNA, labeled with ^{32}P at its 5' (a) or 3' (b) end and treated with DEP, was incubated in the presence of L11, recovered from Mg^{2+} -containing gels (Fig. 1, lanes 4 or 5), and treated with aniline to induce cleavage at modified bases. The resulting fragments were resolved on 20% polyacrylamide sequencing gels. Exclusion of molecules containing chemically altered bases from RNA complexed with L11 (lanes 1) and simultaneous enhancement of the content of modified bases in the unbound RNA (lanes 2) can be inferred from the vastly different band intensities in the two populations (solid dots). Instances where treatment with DEP did not bring about a reproducible difference ($> 50\%$ in both 5' and 3' labeled RNAs as judged by visual inspection) are indicated by arrows. The shaded area indicates the region throughout which exclusions and enhancements were reproducibly observed.

resuspended in 1 μ L of water and an equal volume of deionized formamide containing 0.02% bromophenol blue and 0.02% xylene cyanole was added. Aliquots containing approximately equal ($\pm 10\%$) amounts of radioactivity were loaded onto a 20% polyacrylamide sequencing gel (0.4 mm $\times$ 18 cm $\times$ 35 cm) in 8 M urea/TBE and electrophoresis was carried out at 25 mA constant current until the xylene cyanole tracking dye had migrated approximately 32 cm. Autoradiograms were made using Kodak X-Omat AR film and intensifying screens at -70°C .

RESULTS

To assess the feasibility of separating L11-bound RNA from unbound material, uniformly ^{32}P -labeled transcripts were renatured and incubated in the presence or absence of L11 under conditions appropriate for formation of a specific complex, and the samples were run on a 6% nondenaturing polyacrylamide gel in the presence of Mg^{2+} . In the absence of L11 the RNA was generally resolved into two distinct bands (Fig. 1, lane 1). Both major bands were observed over a broad range of RNA

concentrations (0.9–9 μM) and under virtually all conditions of preheating and renaturation and were not affected by the presence of 3–10% glycerol in the loading buffer. Following recovery from the nondenaturing gel and analysis on 6% denaturing 8 M urea gels, both bands had identical electrophoretic mobilities, indicating that one was not simply a breakdown product of the other. Frequently, additional material was evident in minor bands or as diffuse smears throughout the upper portion of the gel. These additional bands were not observed when the RNA was preheated to 95°C for 45 s prior to renaturation.

In the presence of L11, a band characteristic of a protein-RNA complex was observed. Its mobility was slightly retarded relative to the faster migrating band observed in the absence of L11 (Fig. 1; arrow, lane 2). In some instances the upper of the two major bands appeared to react with L11 and migrated with a somewhat retarded mobility (Fig. 2, lane 6), but in most cases appeared to form an aggregate in the presence of L11 and remained in the well of the gel (Figs. 1–2). The amount of L11-dependent aggregate was only slightly reduced in the presence of glycerol.

Figure 4. Autoradiogram of aniline-induced fragments from free and L11-bound RNA, previously treated with hydrazine. RNA, labeled with ^{32}P at its 5' (a) or 3' (b) end and treated with 25% hydrazine, was incubated in the presence of L11, recovered from Mg^{2+} -containing gels (Fig. 2, lanes 6 or 7), and treated with aniline to induce cleavage at modified bases. The resulting fragments were resolved on 20% polyacrylamide sequencing gels. Instances of exclusion in L11-bound material (lanes 1) and enhancement in free RNA (lanes 2) are indicated by solid dots. Cases where treatment with hydrazine did not bring about a reproducible difference are indicated by arrows. The shaded area indicates the region throughout which exclusions and enhancements were reproducibly observed.

To characterize the content of modified bases in L11-bound material that had been treated with DEP prior to complex formation, ^{32}P -end labeled RNA was incubated with or without L11 and the samples were run on Mg^{2+} -containing gels. Without L11, the chemically modified RNA was again predominantly found in two bands (Fig. 1, lane 3); however, in some instances more material migrated in the upper of two main bands and in the upper portion of the gel relative to untreated RNA (Fig. 1, lane 3). When the contents of modified bases in the two major bands were examined, no differences were detected.

Initial trials using protein L11 and RNA that had been treated with 1 μL DEP for 90 s at 90°C showed that no complex between the modified RNA and L11 could be detected on the gel (data not shown). However, when the amount of DEP was reduced by half and the time of treatment reduced to one min, retarded bands corresponding to the L11 complex were observed for both 3' (Fig. 1, lane 4) and 5'-labeled (Fig. 1, lane 5) RNA.

Treatment of transcripts containing the L11 binding site with 50% hydrazine for 10 min also prevented complex formation (data not shown). When the time of modification was reduced to 5 min, a trace amount of L11-bound material was evident (Fig. 2, lane 2). When the concentration of hydrazine was reduced to 25% and the length of treatment shortened to 5 min, or when 50% hydrazine was used for only 2.5 min, higher levels of complex formation were observed (Fig. 2, lanes 3–4). To ensure recovery of sufficient material for analysis, subsequent experiments characterizing the content of modified uridines were carried out using 25% hydrazine for 5 min.

Similar to unmodified material, hydrazine-treated, ^{32}P -end labeled transcripts migrated primarily as two bands in the absence of L11 on Mg^{2+} -containing gels (Fig. 2, lane 3). In the

presence of L11, retarded bands were observed at the expected positions for both 3'-(Fig. 2, lane 6) and 5'-(Fig. 2, lane 7) labeled samples. In some, but not all, instances another retarded band was evident just above the upper of the two main bands seen in the absence of L11 (see Fig. 2, lane 6).

The content of modified bases in material eluted from relevant bands in the Mg^{2+} -containing gel was determined by treatment with aniline followed by resolution of the resulting fragments on polyacrylamide sequencing gels. Most such fragments corresponded to cleavage at adenosine or guanosine residues in DEP-treated material (Fig. 3) and at uridine nucleotides in hydrazine-treated RNA (Fig. 4). However, unexpected cleavages, especially at labile pyrimidine-adenosine phosphodiester bonds, were frequently observed. In experiments using hydrazine-treated material, a pair of very intense bands corresponding to residues A1086-G1087 was apparent, especially for 3' labeled samples (Fig. 4b). We note that these anomalous bands were only observed when the RNA was recovered from Mg^{2+} -containing gels, perhaps reflecting binding of Mg^{2+} to this region of the free RNA.

When the contents of modified bases in protein-bound (arrows, Figs. 1–2) and unbound (band directly below the L11-specific material, Figs. 1–2) RNA were compared, a striking exclusion of chemically altered bases was apparent throughout the region corresponding to positions 1055–1105 in 23S rRNA for both DEP- and hydrazine-modified samples (shaded region, lanes 1a and 1b, Figs. 3–4). At the same time, the intensities of bands in material not bound to L11 were correspondingly enhanced (lanes 2a and 2b, Figs. 3–4).

Intensities of bands in the autoradiogram that were reduced in the L11-specific material and enhanced in that unable to bind L11 were somewhat variable from experiment to experiment. However, the extent of exclusion was generally greater than 50%, as judged by visual comparison to corresponding bands in the unbound RNA. Nucleotides that were reproducibly excluded in modified form from L11 complexes (solid circles, Figs. 3–4) are indicated as open letters on a model of secondary structure for this region in Figure 5. For ten residues within the 1055–1105 region (U1060, U1065, A1067, G1071, U1078,

Figure 5. Summary of effects of chemical modifications on L11 binding. The sequence of a portion of pPS2 transcripts that contains the L11 binding site is arranged in a secondary structure adapted from Egebjerg *et al.* (13); the 42 base 5' flanking sequence is GGGAAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCC; the 18 base 3' flanking sequence is GGGTACCGAGCTCGAATT. Instances where chemical modification resulted in reproducible exclusion of molecules containing modified bases from L11-bound material and simultaneous enhancement in free RNA (Figs. 3–4) are indicated by open large letters; those where no reproducible interference with complex formation as a result of modification was seen are designated in solid bold letters; cytidines that were not modified in our experiments and bases that could not be clearly resolved on our gels are indicated by small letters.

Figure 6. Region of a proposed tertiary structure (13) required chemically intact for interaction with L11. Sites where chemical modification interfered with L11 binding (black) and those that did not substantially inhibit complex formation (crosshatched) are depicted diagrammatically on a previously proposed tertiary structural model for the L11 binding site (13). A region encompassing several sites where chemically altered bases affected binding is depicted by light stippling.

A1080, A1084, G1093, A1095, and U1101), we reproducibly saw little (<50%) or no evidence of interference with binding by a modified base (arrows in shaded region, Figs 3–4; bold letters, Fig. 5).

DISCUSSION

Comparison to other studies on the L11 binding site

Chemically modified bases were absent from L11-bound RNA in the region corresponding to residues 1055–1105 (shaded regions, Figs. 3–4). These data correspond well to protection studies (12,13) and identification of minimum-sized fragments capable of binding L11 (14), in which bases 1051–1108 were implicated in specific interaction with L11. No exclusion or enhancement of modified bases were observed outside this region, either in the flanking sequences of rRNA or in those from the vector. Our results confirm the approximate size of the L11 binding site, and illustrate that residues scattered throughout the entire region are required intact for binding.

The tertiary structural model of this portion of 23S rRNA presented by Egebjerg *et al.* (13), supported in part by the importance of a base pair between residues 1082 and 1086 (18), suggests a possible mode of binding by L11, which may account for the large number of residues we observed to be important for binding (Fig. 6). Specifically, parts of L11 may interact inside the cavity created by the juxtaposition of loops D and B, bordered by helices 3 and 4, with helix 2 at the base (Fig. 6). Any chemically modified base that prevents formation of this cavity would interfere with complex formation; similarly, any altered residue that disrupts a point of contact with L11 would prevent binding.

Significantly, there were a number of nucleotides scattered throughout the L11 binding site, which had no detectable effect on protein binding when modified. Despite the fact that carbethoxylation of these purines or excision of the corresponding uridines did not appear to interfere with binding, seven of the ten (U1060, U1065, G1071, U1078, A1080, G1093, and U1101) were shown to be protected from chemical or enzymatic probes by the presence of L11 bound to 23S rRNA (13). This is not surprising in view of the differences between the experimental approaches. That is, a given residue may be protected from attack by the presence of bound L11, but may not itself be involved in complex formation.

Our results are also in accord with the notion that residues not directly involved in binding L11 (present study) and not significantly protected from chemical or enzymatic probes by L11 (13) may be required for other functions associated with this region of 23S rRNA. As a specific example, residue A1067, which did not appear to interfere with complex formation with L11, has previously been shown to be the site of methylation in the thiostrepton producing organism *Streptomyces azureus*, rendering its ribosomes completely resistant to the antibiotic (30). Substitution of a pyrimidine at this site also blocks binding by thiostrepton (31).

Similarly, A1095 did not interfere with binding of L11 when modified and was not protected by the presence of L11 alone (13), yet was protected from chemical probes by L11 and thiostrepton together (5). These observations are consistent with the proposed binding by at least part of L11 inside the cavity that is formed on juxtaposition of loops B and D (13, Fig. 6). Thiostrepton could then extend across the top of the L11-filled

cavity, making contact with both L11 and bases within the two loops. According to this model, residues A1067 (loop B) and A1095 (loop D), which face each other across the top of the cavity, would be expected to be protected by bound thiostrepton, but would not be required intact for L11 binding. Consistent with the above discussion, altering residues 1067 or 1095 had only minor effects on binding by L11, but significantly reduced interaction with thiostrepton (19).

Our results are consistent with those of Ryan *et al.* (19), in which changing the identity of any one of several bases throughout the L11 binding site, including most of the residues we observed to be required chemically intact, somewhat reduced the equilibrium constant. However, most of their reported changes did not have dramatic effects on binding by L11, in contrast to our experiments in which the interaction was sufficiently disrupted to prevent comigration of RNA containing a chemically modified base with L11 on Mg^{2+} gels. Based on our experiments alone, we cannot quantify the effect of a single modification on the binding constant or on the lifetime of the complex. We also note that the two experiments are difficult to compare directly because substituting one base for another is substantially different from adding a bulky carbethoxy group or excising the base altogether.

Such differences in experimental approach may also explain why changing the identity of three bases that we found to have no detectable effect on binding when modified (U1060, U1078, and G1093) also reduced the L11 equilibrium constant by a factor of four (19). In the one instance where a single base change resulted in a greater than ten-fold decrease in the binding constant, A1085>U (19), we also observed exclusion of the corresponding carbethoxylated residue from L11-bound material.

It is important to note that in our damage-selection experiments we cannot say whether alteration of a single nucleotide was sufficient to prevent complex formation or whether it prevented the complex from remaining intact during electrophoresis. Neither can we discriminate between residues required intact for proper folding of the RNA prior to interaction with the protein from those involved in a protein-induced structural rearrangement.

Comparison to other damage-selection experiments

Treatment of the L11 binding site with either DEP or 50% hydrazine under conditions similar to those originally described for sequencing RNAs (20) substantially inhibited complex formation. Similar observations have been made previously in damage-selection experiments examining S8/16S rRNA (21), L18/5S rRNA (D.L.Thurlow and R.A.Zimmermann, manuscript in preparation) and L1/L1-specific RNA interactions (P.B.F.Cahill, D.L.Thurlow and R.A.Zimmermann, manuscript in preparation). For both reagents, reducing the concentration of the chemical modifier and/or the time of incubation permitted detection of protein complexes on Mg^{2+} -containing gels. Our interpretation is that chemical modification of any one of many residues in the protein binding site was sufficient to interfere with complex formation or stability.

In the present set of experiments, we were not able to discriminate between modified bases that prevent proper folding from those involved in direct contact with L11. In contrast, both the L1 binding site (P.B.F.Cahill, D.L.Thurlow and R.A.Zimmermann, manuscript in preparation) and 5S rRNA (D.L.Thurlow and R.A.Zimmermann, manuscript in preparation) migrated as well resolved electrophoretic conformers on Mg^{2+} -containing gels, permitting discrimination between

nucleotides that, when modified, interfered with proper folding of the RNA from those that interrupted direct contact with the protein. In both the L1 and L18 binding sites, the majority of bases that interfered with binding were required for proper folding of the RNA, and only a few (three to four) were likely candidates for points of direct contact with the protein.

In our study, the two bands observed in the absence of L11 were not native and partially denatured forms, similar to A and B conformers of 5S rRNA (32) or conformers 1 and 2 of the L1 binding site (P.B.F.Cahill, D.L.Thurlow and R.A.Zimmermann, manuscript in preparation), because molecules containing modified bases were not excluded from the lower (native) band, which formed L11 complexes. Consequently, we identify bases required chemically intact for complex formation with L11, but cannot say whether they were required for the RNA to fold into its native conformation or for direct contact with the protein. If adoption of a tertiary structure such as that proposed by Egebjerg *et al.* (13) is required, it is likely that alteration of any one of many nucleotides within the L11 binding site would interfere with the necessary folding process.

The existence of the upper main band (dimer?), as well as additional minor bands, on the Mg^{2+} -containing gel in the absence of L11 constitutes strong evidence for multimolecular aggregates in our system. Such aggregates are not necessarily intrinsic to the L11 binding site, because the flanking sequences on our transcripts are relatively large and may contribute to RNA/RNA interactions. For example, the presence of several consecutive G residues in the flanking region may result in formation of a K^+ -stabilized aggregate similar to that recently reported for an oligonucleotide derived from 5S rRNA from *E.coli* (33).

Similar bands composed of multimolecular aggregates have been observed on Mg^{2+} -containing gels with substrates for hammerhead ribozymes (34). Such RNA/RNA interactions, and possibly non-specific protein/RNA interactions (Figs. 1–2, material remaining in the wells in the presence of L11), raise potential problems in characterizing thermodynamic and kinetic parameters using experimental approaches such as filter binding assays alone. We suggest that it would be useful to report the behavior of the RNA under investigation on Mg^{2+} gels to facilitate interpretation of filter binding assays.

The set of protein-RNA interactions examined to date using damage-selection provide some insight into general mechanisms underlying different types of RNA-protein interactions. In each instance involving ribosomal proteins and RNA, any one of many nucleotides, when chemically altered, was sufficient to interfere with binding. Since only a small fraction of such residues had been implicated in direct contact with the protein, adoption of a very specific, and easily disrupted, three-dimensional conformation must have been essential. The protein may play an important role in stabilizing one of several possible tertiary structural arrangements.

In contrast, when the interaction between tRNAs and the enzyme nucleotidyltransferase was studied, only a small number of residues, clustered at the corner of the tRNA's three-dimensional structure or near its 3' end, were required chemically intact (23). These two sets of interactions are fundamentally different in that ribosomal protein-RNA complexes are stable over long periods of time, whereas the enzyme-substrate interaction is interrupted quickly following catalysis. Perhaps having few sites of interaction is most appropriate for RNA-enzyme

interactions, where disruption of one as a result of catalysis can lead to immediate release of product. For more long-lived complexes, multiple sites of interaction would stabilize the interaction.

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