Nucleic Acids Research, 1994, Vol. 22, No. 9 1687–1695

The binding site for ribosomal protein S8 in 16S rRNA and *spc* mRNA from *Escherichia coli*: minimum structural requirements and the effects of single bulged bases on S8 – RNA interaction

Herren Wu, Lihong Jiang and Robert A.Zimmermann*
Department of Biochemistry and Molecular Biology and Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, MA 01003, USA

Received December 22, 1993; Revised and Accepted March 28, 1994

ABSTRACT

Through specific interactions with rRNA and mRNA, ribosomal protein S8 of *Escherichia coli* plays a central role in both assembly of the 30S ribosomal subunit and translational regulation of *spc* operon expression. To better understand S8 – RNA association, we have measured the affinity of S8 for a number of variants of its rRNA and mRNA binding sites prepared by *in vitro* transcription or chemical synthesis. With the aid of site-directed deletions, we demonstrate that an imperfect, 33-nucleotide helical stem encompassing nucleotides 588 – 603 and 635 – 651 possesses all of the structural information necessary for specific binding of S8 to the 16S rRNA. This segment consists of two short duplexes that enclose a conserved, assymetric internal loop which contains features crucial for protein recognition. The S8 binding site in *spc* operon mRNA is very similar in both primary and secondary structure to that in 16S rRNA except for the presence of two single bulged bases in one of the duplex segments. In addition, the apparent association constant for the S8 – mRNA interaction is approximately fivefold less than that for the S8 – rRNA interaction. We show that the difference in affinity can be attributed to the effects of the bulged bases. Deletion of the bulged bases from the mRNA site increases its affinity for S8 to a level similar to that of the rRNA, whereas insertion of single-base bulges at equivalent positions within the rRNA site reduces its affinity for S8 to a value typical of the mRNA. Single-base bulges in the proximity of essential recognition features are therefore capable of modulating the strength of protein – RNA interactions.

INTRODUCTION

Protein–RNA interactions play a fundamental role in the assembly, maturation and function of the *Escherichia coli* ribosome (1,2). Associations involving protein S8 are of particular interest because S8 not only binds to 16S rRNA early in 30S subunit assembly but, in addition, serves as a translational repressor of *spc* operon mRNA, which encodes S8 and 10 other ribosomal proteins (3,4). The S8 binding site in 16S rRNA, initially delineated by sequence analysis of rRNA fragments protected against RNase digestion by bound protein, is located between nucleotides 583 and 653 within the central domain and consists of a long hairpin structure interrupted by two internal loops (5–7). The interaction of S8 with this region has been further defined by comparative sequence analysis (8,9), modification-interference experiments (10), and an investigation of its susceptibility to chemical modification and nuclease attack in the presence and absence of S8 (11,12). Moreover, S8 has been chemically cross-linked to three different oligonucleotides within this segment (13). Both site-directed and naturally occurring base replacements indicate that the phylogenetically conserved nucleotides at positions 595–598 and 640–644 comprise the crucial recognition features for S8 binding (9,14,15). Although the arrangement of these bases varies somewhat in different secondary-structure models (9,11,16), it is clear that they are part of a significant structural irregularity in an otherwise regular, base-paired stem. In the present report, we depict this feature as a small, asymmetric internal loop.

Despite the evident importance of the conserved internal loop in S8–rRNA interaction, there is little information available on the role of the surrounding portions of the S8 binding site. RNase digestion experiments showed that rRNA fragments encompassing nucleotides 583–605 and 631–653 were most strongly protected by S8, while nucleotides 624–630 were only weakly protected and nucleotides 606–623 were not protected at all (7). These results imply that the apical stem-loop and the internal loop adjacent to it may not be required for S8 recognition, particularly as they are not phylogenetically conserved (7,8). However, the replacement of G627 by A, which is expected to disrupt base pairing in the apical helix, severely impaired S8 binding (9), suggesting that this feature plays a role in S8 association. To determine the portions of the sequence between

*To whom correspondence should be addressed

nucleotides 583 and 653 that are essential for S8 binding, we have used a series of deletions to map the S8 binding site in greater detail. Variants of rRNA fragments containing the small internal loop were prepared by *in vitro* transcription of synthetic DNA templates with T7 RNA polymerase (17) or by chemical synthesis, and their capacity to associate with S8 was assessed by a filter-binding assay (18). We conclude that the minimum structure required for high-affinity binding of S8 consists of the imperfect helix spanning nucleotides 588–603 and 635–651. A similar conclusion has recently been reached by Mougel and co-workers (15).

Comparison of the S8 binding site in *spc* operon mRNA with that in 16S rRNA revealed striking similarities in both primary and secondary structure, indicating that S8 associates with structurally analogous sites in both RNAs (9,19). Nonetheless, the apparent association constant for the S8–mRNA interaction is almost an order of magnitude less than that for the S8–rRNA interaction (9). We proposed that this difference may due to the effects of two single bulged bases that are present in the mRNA but not in the rRNA, and which represent the only significant discrepancies between two S8 binding sites (9). To evaluate the role of the two bulged bases in S8–RNA interaction, rRNA fragments with bulged bases inserted at equivalent positions and mRNA fragments from which the bulged bases had been deleted were tested for their S8-binding capacity. From the measured association constants, we show that single bulged bases are indeed capable of modulating the affinity of RNA for S8.

MATERIALS AND METHODS

Materials

T7 RNA polymerase (200 units/ml) and T4 polynucleotide kinase were purchased from Epicentre Technologies. RNasin ribonuclease inhibitor was obtained from Promega. 1 M tetrabutylamonium fluoride in tetrahydrofuran (TBAF/THF) reagent was from Aldrich. [α-^{32}P]UTP ($\geq$3000 Ci/mmol) and [γ-^{32}P]ATP ($\geq$6000 Ci/mmol) were provided by New England Nuclear. The C-4 HPLC column was purchased from Vydac, the Synchropak RP-P C_{18}-silica column was obtained from Symchrom, and the Nucleogen DEAE 60-7 HPLC column was supplied by Chemical Dynamics Corp. DE52 ion exchange medium was from Whatman. Oligodeoxyribonucleotides were produced by the DNA Synthesis Facility at the University of Massachusetts, Amherst.

In vitro transcription of RNA

Fragments of 16S rRNA and *spc* operon mRNA were transcribed *in vitro* from either plasmid or synthetic DNA templates using T7 RNA polymerase. Transcripts from plasmid pEX0 were prepared and labeled with ^{32}P as described by Gregory *et al.* (9). Transcription of synthetic DNA was performed according to Milligan and Uhlenbeck (17). In the latter procedure, single-stranded DNA templates containing the T7 ϕ10 promoter and a sequence encoding the desired RNA segment were deblocked and purified by electrophoresis through 8 to 12 % polyacrylamide gels (acrylamide:*N,N'*-methylenebisacrylamide, 19:1, w/w) in TBE buffer (100 mM Tris, 100 mM boric acid, pH 8.3, and 2.5 mM EDTA) containing 8 M urea. DNA fragments were detected by ethidium bromide staining or by UV shadowing, excised from the gel and extracted into a solution containing 0.5 M NH_4OAc, 0.01 M $Mg(OAc)_2$, 0.1 mM EDTA and 0.1% SDS by shaking overnight at 37°C. The templates were then precipitated with ethanol, and resuspended in TE buffer (10 mM Tris–Cl, pH 8.0, and 1 mM EDTA). To prepare substrates for transcription, an equimolar amount of a 17-nucleotide DNA fragment complementary to the T7 promoter was annealed to the template strand by incubation at 65°C for 5 minutes, followed by slow cooling to room temperature over 15 minutes. Transcription reactions were carried out in 50 ml of 40 mM Tris–Cl, pH 7.5, 10 mM NaCl, 6 mM $MgCl_2$ and 10 mM dithiothreitol containing 0.5 nmol of annealed templates, 50 nmol of each ribonucleoside triphosphate, 50 μCi of [α-^{32}P]UTP, 700 units of T7 RNA polymerase, 40 units of RNasin ribonuclease inhibitor and 50 mg/ml BSA, at 37°C for 2 hours. The ^{32}P-labeled RNA fragments were purified by denaturing polyacrylamide gel electrophoresis, recovered as above and resuspended in TE buffer. The RNA transcripts used in this investigation are depicted in Figures 1 and 4. The tetra-loop GC-AA was incorporated into several of the RNAs to stabilize their secondary structure, and certain alterations were made at the 5′ and 3′ ends of the RNA to increase the efficiency of transcription. In several cases, the primary structure of the transcribed fragments was verified by sequence analysis, using the enzymatic method of Donis-Keller (20).

Chemical synthesis of RNA

RNA fragments corresponding to the 5′ or 3′ portions of the S8 binding site were synthesized chemically by the DNA Synthesis Facility at the Thomas Jefferson University or by the W.M.Keck Biotechnology Resource Laboratory at Yale University. Approximately 1 μmol of each RNA was synthesized and provided in a 2′-O-silylated form. The 2′-OH groups were deprotected by treating the RNA fragments with 1 M TBAF in THF, after which they were purified by HPLC (21). The RNA was applied to a Vydac C-4 column (4.6 mm×25 cm) and eluted at room temperature using a linear gradient of 5–40% acetonitrile in 0.1 M triethylammonium acetate, pH 6.8. This step separated deprotected RNA fragments from those which were only partially deprotected. Fractions containing the former were pooled, partially dried and loaded onto a Nucleogen DEAE 60-7 column (4×125 mm), and the RNA fragments were eluted with a linear gradient of 0–1.5 M KCl in 20 mM NaOAc, pH 6.5, and 20% acetonitrile. Aliquots from the peak fractions were labeled with ^{32}P at the 5′ end using T4 polynucleotide kinase and [γ-^{32}P]ATP (22), and checked for size and homogeneity by electrophoresis on 20% polyacrylamide gels containing 8 M urea. Full-length RNA fragments were pooled, desalted either by dialysis against distilled H_2O or by chromatography on a DE52 ion exchange cloumn using triethylammonium bicarbonate, pH 7.5, as the eluant. The purified, desalted RNA fragments were lyophilized and stored at −20°C. The structures of the synthetic RNA fragments are illustrated in Figure 3.

Protein purification

Protein S8 was overexpressed from plasmid pET-*rpsH* in BL21(DE3)/pLysS cells (23), isolated in the form of inclusion bodies, and extracted with 6M urea as described by Wu *et al.* (24). The urea extract was loaded onto a Synchropak RP-P C_{18}-silica column (6.5 mm silica, 300-Å pore, 4.1×250 mm), and eluted with a 25–60% acetonitrile gradient in 0.1% trifluoroacetic acid. Proteins were identified by electrophoresis through 15% polyacrylamide gels in the presence of 0.1 % sodium dodecyl sulfate (25). The concentration of purified S8

was determined by the Bradford method using BSA (fraction V) as a standard [26; see also ref. 24)].

S8–rRNA and S8–mRNA interaction

The S8-binding ability of synthetic rRNA and mRNA fragments was determined by a quantitative nitrocellulose filter-binding assay (18,24). In assays of S8–rRNA interaction, the protein and the RNA fragments were incubated separately in TMK–Cl buffer (50 mM Tris–OAc, pH 7.6, 20 mM $Mg(OAc)_2$, 350 mM KCl, and 5 mM β-mercaptoethanol) for one hour at 40°C. A fixed amount of ^{32}P-labeled rRNA fragments (0.01 to 1 pmol) was then mixed with increasing amounts of S8. The mixtures were incubated for 10 minutes at 40°C, followed by 15 minutes at 0°C, and filtered through nitrocellulose membranes. The RNA retained on the filter in the form of S8–RNA complexes was quantitated by Cerenkov counting. Each data point represents an average of three individual measurements. Assays of S8–mRNA interaction were carried out in an identical manner except that TMK–OAc buffer (50 mM Tris–OAc, pH 7.6, 20 mM $Mg(OAc)_2$, 350 mM KOAc, and 5 mM β-mercaptoethanol) was used instead of TMK–Cl buffer. Chemically synthesized rRNA fragments were first labeled with ^{32}P at the 5′ end and purified by polyacrylamide gel electrophoresis. rRNA13a:rRNA13b and rRNA14a:rRNA14b were annealed in TMK–Cl buffer at 65°C for 5 minutes and cooled at room temperature for 15 minutes prior to incubation with protein S8 and measurement of protein–RNA interaction.

RESULTS

Interaction of S8 with rRNA transcripts

Studies performed with intact *E.coli* 16S rRNA a decade or more ago established that the binding site for ribosomal protein S8 is located within a segment encompassing nucleotides 583–653 which folds into a long, imperfect stem-loop structure designated helix 21 (5–7; see ref. 27 for numbering of helices in 16S rRNA). Subsequently, it was shown that *in vitro* transcripts containing residues 584 to 756 or 435 to 708 exhibited almost the same affinity for S8 as intact 16S rRNA (9,11). To further characterize the structural features of the 16S rRNA that mediate S8 recognition and interaction, we prepared a number of variants of the S8 binding site by *in vitro* transcription of synthetic DNA templates and measured their affinities for S8 by a nitrocellulose filter assay. The fragment used as a benchmark in these experiments was a 74-nucleotide transcript (rRNA1) that spans all of helix 21 together with a five base-pair extension at its base that was designed to confer additional stability. As illustrated in Figure 1, this structure consists of three duplex segments, 21a, 21b and 21c, interrupted by two internal loops and capped by a five-residue apical loop. Association of S8 with the 74-nucleotide fragment was characterized by roughly the same binding constant as for the 299-nucleotide transcript (Table 1), indicating that it contained most, if not all, of the structural determinants required for specific interaction with S8.

Truncation of the S8-binding helix

Apical portions of helix 21 were systematically deleted as shown in Figure 1 (rRNA2–5). Association constants for the interaction of S8 with the corresponding transcripts are listed in Table 1 and typical binding curves are presented in Figure 2. An association constant at least 50% as great as that of rRNA1 was taken to represent full binding activity. As neither partial (rRNA2) nor complete (rRNA3) removal of helix 21a had a significant effect on complex formation, this feature is evidently not essential for S8 binding. In addition, the rearrangement of the larger internal loop that occurs in rRNA3 argues against a role for the corresponding bases in S8–rRNA association. The three apical base pairs of helical segment 21b can also be deleted with only

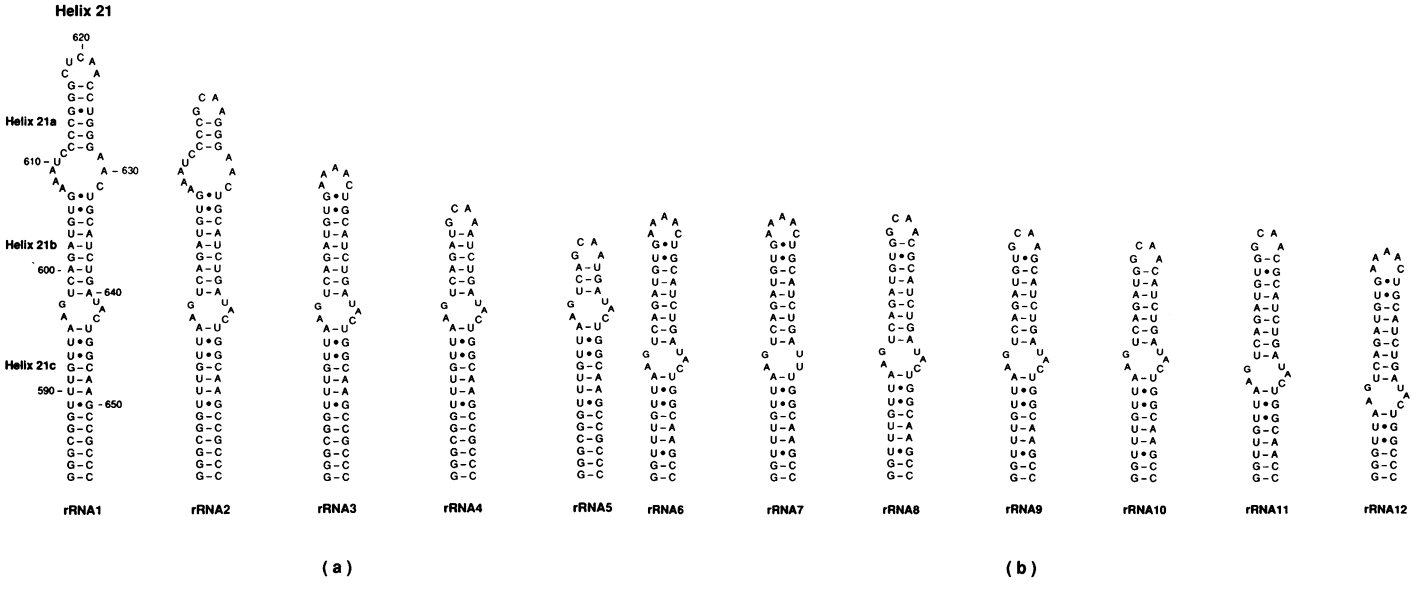

Figure 1. Secondary structure of rRNA transcripts. The tetraloop, GCAA, was used as the apical loop in several of the rRNA transcripts for stability. Several G–C pairs were added to the base of the helix 21c to facilitate transcription. rRNA1: benchmark transcript; rRNA2–5: stepwise apical truncations of helix 21; rRNA6: base of helix 21 truncated by four base pairs; rRNA7: residues A642 and C643 in rRNA6 replaced by U; rRNA8; apical loop of rRNA6 replaced by tetraloop and U632 changed to C; rRNA9–10: apical truncations of rRNA8; rRNA11: basal G-C pair removed from rRNA8 and U589–G650 changed to G–C; rRNA12: three basal pairs removed from rRNA6 and two U–A pairs changed to G–C.

Table 1. Apparent association constants for the interaction of RNA variants with protein S8

RNA	Size of rRNA (nts)	Association constant[b] K'_a (M^{-1}, $\times 10^{-6}$)	K'_a (mutant) / K'_a (wild-type)
pEX0[a]	299	8.2	1.44
rRNA1	74	5.7	1.0
rRNA2	67	4.4	0.77
rRNA3	54	4.6	0.81
rRNA4	47	2.9	0.51
rRNA5	41	1.2	0.21
rRNA6	46	4.0	0.70
rRNA7	45	0.1	0.02
rRNA8	45	2.9	0.51
rRNA9	43	2.4	0.42
rRNA10	41	2.3	0.40
rRNA11	43	0.1	0.02
rRNA12	40	0.5	0.09
rRNA13a:13b	39	14.0	2.46
rRNA14a:14b	33	1.5	0.26

[a]RNA transcribed from plasmid pEX0 encompassing nucleotides 435–768 of the 16S rRNA (Gregory *et al.*, 1988).
[b]Apparent association constants for the interaction of protein S8 with rRNA fragments were measured in TMK–Cl buffer.

a small influence on the protein-binding capacity of the transcript. However, removal of the next three base pairs, corresponding to nucleotides 601–603 and 635–637 (rRNA5), led to an 80% decrease in K'_a relative to rRNA1.

Deletion of four of the five base pairs that extend the basal portion of helix 21 produced no major change in the affinity of S8 for the rRNA transcript (compare rRNA3 and rRNA6, Table 1). The binding constants are also relatively insensitive to the nature of the apical loop. Thus, the three different loop sequences used in rRNA1 through rRNA5, including the tetra-loop motif, GCAA, have little influence on S8 binding. This is clearly exemplified by transcript rRNA8, in which the apex of the helix is stabilized by the presence of a tetra-loop and replacement of the apical base pair, G606·U632, with G–C. Here the modified transcript exhibits only a modest decrease in affinity for S8 relative to the very similar structure, rRNA6 (Table 1). Removal of either one or both base pairs from the apical end of helical segment 21b to yield rRNA9 and rRNA10, respectively, confirms that they do not play a critical role in S8 binding (compare with rRNA8, Table 1). By contrast, further modifications of the basal portion of helix 21c were found to have a deleterious effect on S8–rRNA interaction. The S8-binding capacity of rRNA11, in which the entire five base-pair extension was deleted and the U589·G650 base pair was replaced by G–C, dropped to a barely detectable level. Simultaneous removal of the G588–C651 and U589·G650 base pairs and substitution of the U590–A649 and U591–A648 base pairs by G–C (rRNA12) resulted in a tenfold reduction in K'_a relative to rRNA1 (Table 1). The fact that rRNA11 is more severely impaired in its association with S8 than the more extensively altered rRNA12 suggests that the introduction of a G–C pair in place of U589·G650 may actually antagonize S8 binding.

To demonstrate that the stem-loop structures derived from helix 21 do not require a covalently closed apical loop for S8 recognition, the S8 binding site was reconstructed from pairs of shorter fragments representing its 5′ and 3′ segments (Figure 3a). One of these, rRNA13a:13b, corresponds closely to rRNA9 except that the U605·G633 base pair was changed to C–G in order to stabilize the end of the apical stem. Although neither strand exhibited any interaction with S8 individually, the duplex proved to be an excellent substrate for S8 binding and was characterized by a K'_a of 1.4×10^7 M^{-1} (Table 1 and Figure 3b). The fact that the affinity of S8 for rRNA13a:13b is higher than that for comparable continuous transcripts (e.g., rRNA3, rRNA6, rRNA9) indicates that deletion of the apical loop may remove a constraint on S8–rRNA interaction. A second fragment, rRNA14a:14b, lacks additional base pairs at both the apical and basal ends of the stem-loop structure. Its affinity for S8, while substantial, was almost an order of magnitude less than that of rRNA13a:13b, suggesting once again that deletion or modification of the U589·G650 base pair may impede protein binding.

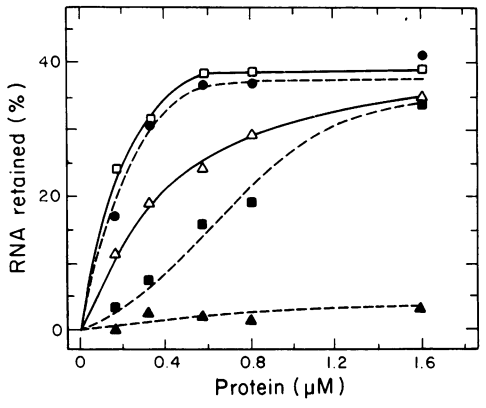

Figure 2. Binding curves for the interaction of S8 with several rRNA transcripts. ^{32}P-labeled rRNA fragments were synthesized by *in vitro* transcription, purified by gel electrophoresis and assayed for association with S8 by the filter-binding technique as described in Materials and Methods. Plasmid pEX0 was transcribed to yield a 299-nucleotide rRNA fragment (9). Values of RNA retained on the filter in the absence of protein (3–5%) were subtracted before the data were plotted. pEX0 rRNA (□); rRNA1 (●); rRNA4 (△); rRNA5 (■); rRNA7 (▲).

Together, our results indicate that the imperfect stem-loop encompassing residues 588–603 and 635–651, a total of 33 nucleotides, possesses all of the structural information necessary

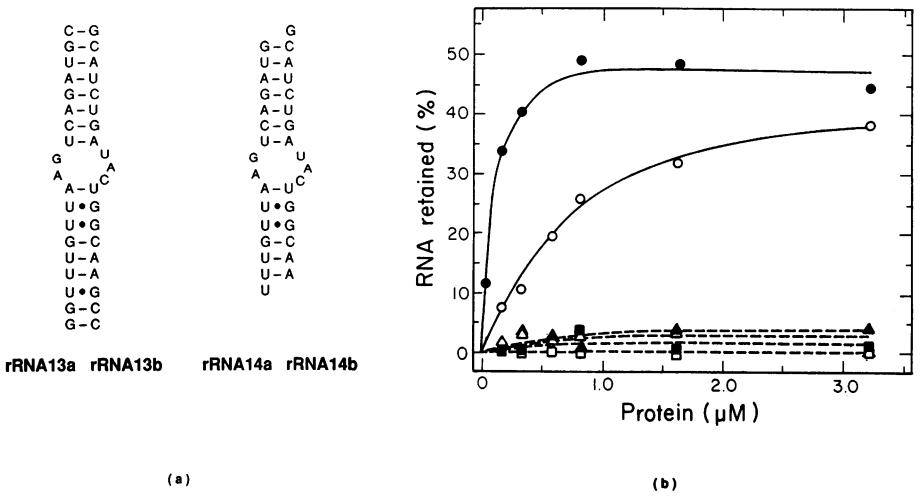

Figure 3. Reconstitution of the S8 binding site from separate rRNA fragments. (**a**) Secondary structure of reconstituted rRNA molecules, rRNA13a:13b and rRNA14a:14b. (**b**) Binding curves for the interaction of the reconstituted rRNA molecules with protein S8. Background values were subtracted prior to plotting. rRNA13a:13b (●); rRNA14a:14b (○); rRNA13a (■); rRNA13b (▲); rRNA14a (□); rRNA14b (△).

for the specific binding of protein S8 at high affinity even though constructs with further truncations of either the apical or basal helices (e.g., rRNA5 or rRNA14a:14b) exhibit significant S8-binding activity as well.

Specificity of S8 interaction with rRNA transcripts

The specificity of the interaction of protein S8 with rRNA transcripts corresponding to portions of helix 21 was tested in two ways. First, a variant of rRNA6, in which the AC dinucleotide sequence at positions 642–643 was replaced by a single U residue (rRNA7), displayed no detectable binding to S8 whatsoever under our conditions (Figure 2). The effect of this substitution, previously shown to abolish the RNA-binding capacity of the 299-nucleotide fragment transcribed from plasmid pEX0(9), confirms that S8 recognizes specific structural features in the transcripts and, at the same time, underscores the essentiality of the small internal loop for S8–rRNA interaction. In a second experiment, a mutant of protein S8, SG78, which is unable to associate with the 299-nucleotide transcript (H.Wu, I.Wower and R.A.Zimmermann, unpublished) also failed to interact with rRNA3. This observation demonstrates that the rRNA fragments described here did not simply bind to basic proteins in a nonspecific manner. In a final control, S8 was found to have no affinity for a synthetic DNA fragment corresponding in sequence to rRNA6.

Anion effects on S8–rRNA interaction

It was noted previously that the affinity of S8 for intact 16S rRNA is strongly influenced by the anion present in the buffer (28). In particular, the apparent association constant was observed to rise approximately one order of magnitude when acetate was substituted for Cl^-. We have noted a similar effect in our experiments with fragments of the 16S rRNA. For example, the association constant for the interaction of S8 with the 299-nucleotide fragment transcribed from pEX0 is 8.2×10^6 M^{-1} in Cl^- and 4.0×10^7 M^{-1} in acetate, a fivefold difference. In the case of rRNA6, however, no significant difference in K'_a was found when Cl^- was replaced by acetate. The decrease in the anion effect with the reduction in the size of the rRNA indicates that it probably arises from an influence on the RNA and not on protein S8, and that it does not play a significant part in S8–rRNA interaction.

The influence of single bulged bases on S8–RNA interaction

In an earlier communication from this laboratory, it was shown that the site through which S8 mediates regulation of *spc* mRNA translation is very similar to the S8 binding site in 16S rRNA, both in overall secondary structure and in the arrangement of several conserved bases implicated in S8 recognition (9). However, in addition to the shared features, our secondary-structure model of the mRNA binding site postulates the presence of two individual bulged nucleotides in the base-paired stem that corresponds to helix 21b in 16S rRNA. At the same time, the affinity of S8 for the mRNA is approximately fivefold less than that for the rRNA. To relate these two findings, we speculated that the lower affinity of the mRNA site for S8 might be attributable to the bulged bases, and that single base bulges might serve to modulate the strength of protein–RNA interaction (9). The experiments described below were designed to test the validity of this hypothesis.

To assess the influence of bulged bases on S8–rRNA interaction, transcripts analogous to rRNA8 were prepared with single-base insertions at positions corresponding to those in the mRNA binding site (Figure 4). These additions did not appear to introduce any new self-complementary segments within the fragment. While the insertion of a C residue between nucleotides 638 and 639 (rRNA15) affected S8 binding only minimally, the introduction of a U between nucleotides 636 and 637 in addition to the C between 638 and 639 (rRNA16) resulted in a sixfold decrease in association constant (Figure 5). The consequences of deleting bulged bases from the mRNA binding site for S8 were examined in a further series of experiments. A template encoding the S8-binding stem-loop-stem structure from *spc* operon mRNA, including the two bulged bases, was first constructed and transcribed to yield mRNA1. Additional transcripts were produced that lacked U952 (mRNA2), C955 (mRNA3), or both (mRNA4) (Figure 4). The results of the S8 binding assays, depicted in Figure 5, show that the deletion of either U952 or C955 leads

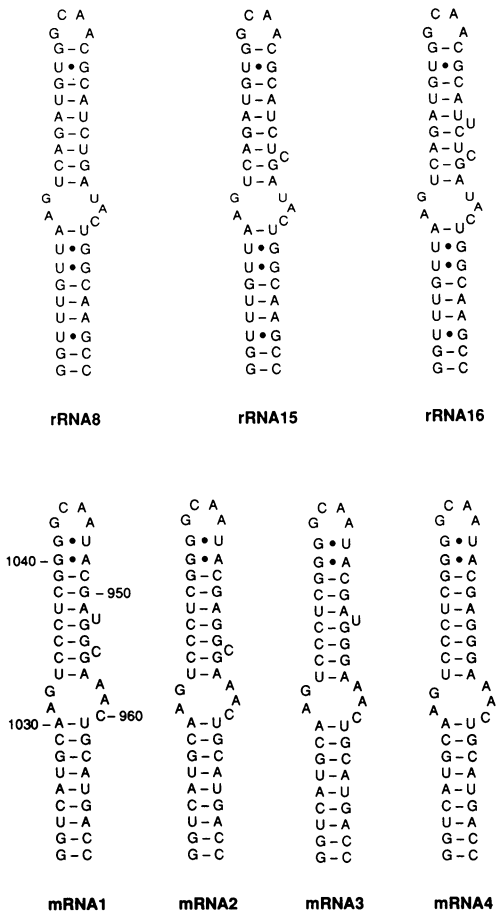

Figure 4. RNA variants used to study the effect of single bulged bases on S8–RNA interaction. rRNA15: C residue inserted between U638 and G639 of rRNA8; rRNA16: U residue inserted between U636 and C637 of rRNA15; mRNA1: stem-loop-stem structure containing the binding site for S8 in *spc* mRNA; mRNA2: U952 deleted from rRNA1; mRNA3: C955 deleted from rRNA1; rRNA4: U952 and C955 deleted from rRNA1.

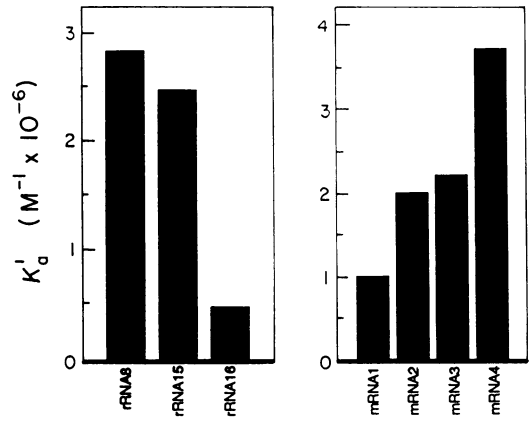

Figure 5. Influence of single bulged bases on S8–rRNA interaction. The association constants (K'_a) for the S8–RNA interactions were obtained from nitrocellulose filter assays as described in Materials and Methods. For structures of the variant rRNA and mRNA binding sites for protein S8, see Figure 4.

to a twofold increase in affinity for S8, whereas the deletion of U952 and C955 together is accompanied by a fourfold rise in the association constant. The magnitude of both the decrease in K'_a that results from the introduction of single-base bulges into the rRNA binding site for S8 and the increase in K'_a that results from the deletion of base bulges from the mRNA binding site for S8 closely parallels the difference in affinity for S8 originally noted in the longer rRNA and mRNA transcripts derived from plasmids pEX0 and pEX*spc* (9). It is therefore evident that single-base bulges can modulate the affinity of S8–RNA interaction.

DISCUSSION

Minimum RNA structure required for interaction with protein S8

Through the use of specific RNA fragments prepared by *in vitro* transcription or by chemical synthesis, we have shown that the 33-nucleotide stem-loop-stem structure composed of residues 588–603 and 635–651 of *E.coli* 16S rRNA is the minimum structure required for the binding of ribosomal protein S8 with near normal affinity (Figure 6). Our benchmark for full binding activity was a 74-nucleotide fragment spanning nucleotides 584 to 651 (rRNA1) which encompassed the region of the 16S rRNA protected from nuclease digestion by bound S8 (9; see Figure 1). Variants that bound S8 with an affinity at least 50% as great as rRNA1 were assumed to contain all of the structural features necessary for interaction with the protein. Analysis of fragments lacking the apical loop, helix 21a, the larger internal loop and the three apical base pairs of helix 21a (rRNA2–4,9,10) demonstrated that none of these features was essential for S8 binding (see Figure 1, Table 1). However, the deletion of additional base pairs from the apical portion of helix 21b (rRNA5) resulted in more than a 50% decrease in S8 affinity. Moreover, while the removal of four of the five extended base pairs adjacent to helix 21c (rRNA6) did not materially impair S8–rRNA interaction, further deletions or alterations within the basal portion of helix 21c (rRNA11,12) caused a drop in the affinity of the RNA for S8 of over 90%. Finally, our results indicate that the loop which closes the apex of the helical stucture plays no role in complex formation since neither the presence of three different loop sequences (rRNA1–3) nor the absence of a closing loop (rRNA13) had a deleterious influence on S8 binding. Using a related strategy, Mougel and co-workers (15) recently concluded that the minimum binding site for protein S8 consists of a 37-nucleotide structure similar to that described here.

While our results strongly suggest that all of the essential determinants of S8 recognition and binding are located within the imperfect helix defined by nucleotides 588–603 and 635–651, two anomalous observations need to be reconciled with the present findings. It has been reported that the chemical reactivity of a number of bases in the regions encompassing positions 570–585, 810 and 855–870 is severely curtailed in the S8–16S rRNA complex (12). Although we have determined that these bases are not necessary for specific S8–rRNA interaction, they may represent secondary contact sites. Alternatively, decreases in nucleotide reactivity may be attributable to S8-induced rearrangements within the 16S rRNA structure. Furthermore, we previously reported that a G to A mutation at position 627, which disrupts the C613–G627 base pair in helix 21a, results in a sharp decline in the S8–rRNA association constant (9). This observation suggested that helix 21a was important for the integrity of the protein binding site. However, since helix 21a can be deleted in its entirety without

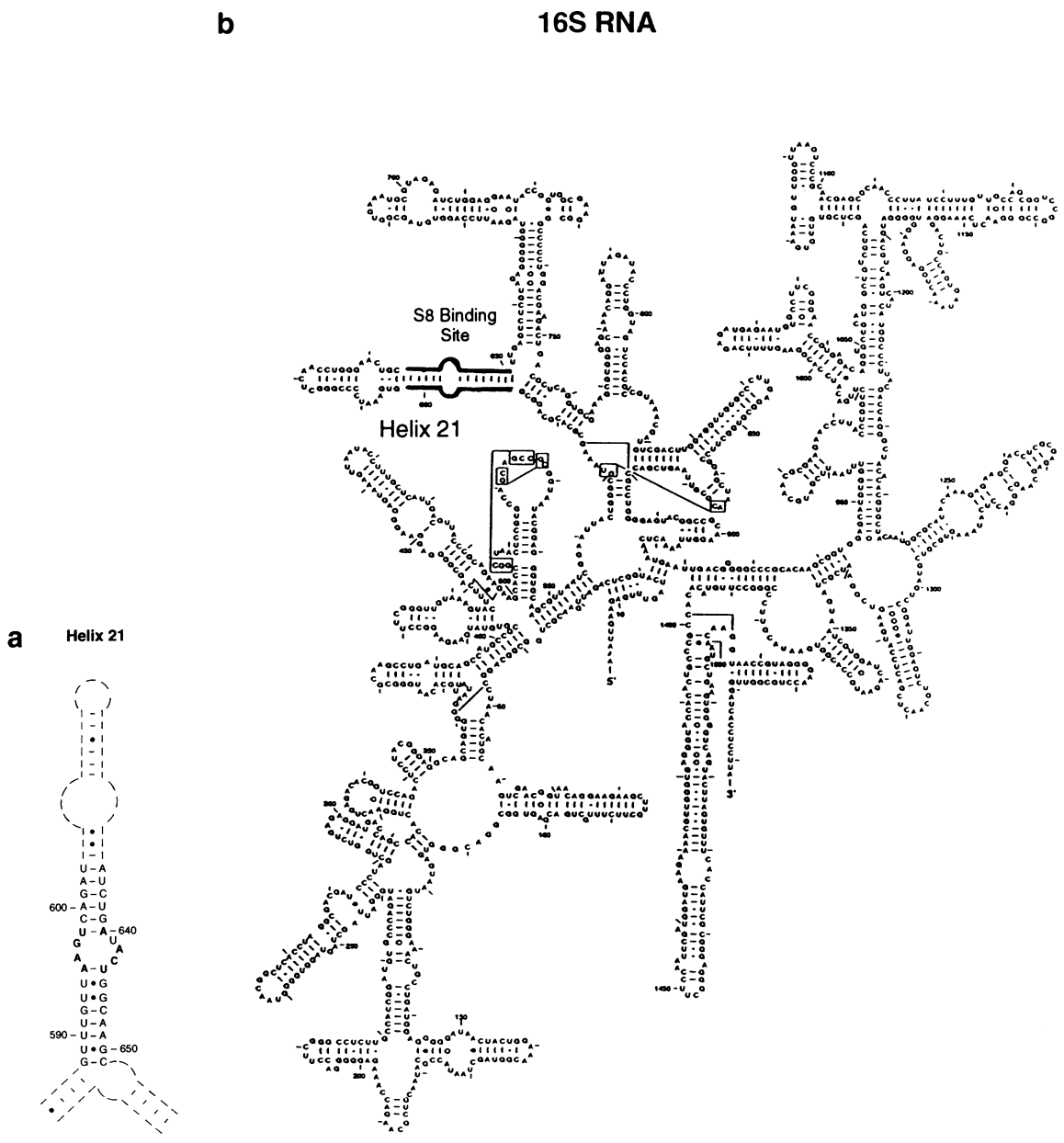

Figure 6. The binding site for ribosomal protein S8. (**a**) Minimum structure within helix 21 of *E.coli* 16S rRNA required for interaction with protein S8. Evolutionarily conserved bases (9) are indicated in boldface. (**b**) The location of the S8 binding site within the secondary structure of 16S rRNA (50) is delimited by heavy lines.

adverse effect on S8−rRNA interaction (rRNA3), it is possible that the G to A transition at position 627 led to the formation of an alternative secondary or tertiary structure that interfered with S8 binding.

Structural determinants of the rRNA binding site for protein S8

Our model of the minimum S8 binding site consists of two short helices that enclose an asymmetric, five-base internal loop composed of nucleotides 596−597 and 641−643. Alternative models of this stem structure, which entail canonical and noncanonical pairing of certain bases within the loop, have been advanced on the basis of phylogenetic comparisons and chemical modification data (11,16). Nonetheless, as recent NMR studies have shown that similar protein recognition sites in other RNA molecules can be configured in unexpected and unpredictable ways (29,30), we prefer to represent this feature as an unpaired loop until a more precise physical description is available. The likelihood that the internal loop is directly involved in S8 recognition was initially inferred from the fact that the loop bases, as well as the adjacent base pairs, are highly conserved in a wide variety of prokaryotic 16S rRNAs capable of binding *E.coli* S8 (8,9). The importance of the bases at positions 641, 642 and 643 in S8−rRNA interaction has been confirmed by site-directed mutagenesis experiments in which single-nucleotide deletions or substitutions reduced the binding constant for S8 by roughly two orders of magnitude (9,15).

While the significance of the small internal loop in S8−rRNA interaction is well documented, the part played by the duplex stems is less clear. Our model of the minimum S8 binding site

includes six of the nine base pairs of helix 21b as well as the eight base pairs of helix 21c. Although the primary role of the helices may be to maintain the essential residues in and adjacent to the internal loop in the correct relative orientation, it is likely that they are in close proximity to the protein. Thus, many of the bases in helices 21b and 21c are resistant to nuclease attack or chemical modification in the presence of S8 (7,11), or interfere with S8 binding when chemically modified prior to interaction (10). Moreover, as shown here, the removal of at least some of the base pairs of the minimum binding site seriously impairs complex formation (e.g., rRNA5,12). Nonetheless, the identity of most of the base pairs is not evolutionarily conserved (8,10), and the replacement of U594·G645 by U−A, or of G604−C634 and G588−C651 by G·U, does not affect S8 binding (9). There are only three conserved base pairs in helices 21b and 21c, G604−C634, U598−A640, and A595−U644, that also occur at equivalent positions in the S8 binding site in *spc* mRNA (9). While the conserved G604−C634 pair is dispensable for high-affinity S8−rRNA interaction, U598−A640 and A595−U644 appear to be essential. Thus, it has been found that the deletion of A595 or A640, as well as the replacement of U598 by A or of A640 by U, results in a sharp decrease in S8 binding activity (15). It is therefore unlikely that S8 makes any base-specific contacts with the helical stems outside the segments encompassing nucleotides 595−598 and 640−644, although it may form specific hydrogen bonds, or nonspecific electrostatic contacts, with the sugar-phosphate backbone in these regions (see ref. 11). Interestingly, substitution of the U589·G650 base pair in helix 21c with G−C (rRNA11) drastically reduces the affinity of the rRNA for S8. While this U·G pair is not itself highly conserved, and is replaced by a U−A pair in *spc* mRNA, comparative analysis reveals a discrimination against G at position 589 in other prokaryotic 16S rRNAs that interact with S8. It is possible that G589 antagonizes complex formation by making an unfavorable contact with the protein.

Numerous protein−RNA interactions are characterized by the involvement of RNA helices with internal loops or bulges (2,31−34), suggesting that they may make use of certain common strategies. Loop and bulge structures are advantageous for specific protein recognition because they distort the sugar-phosphate backbone and provide access to the functional groups of bases normally buried in the narrow major groove of the A-form helix. Two of the most thoroughly investigated interactions are those of the HIV Rev and Tat proteins with the RRE and TAR elements, respectively, in HIV RNA. The high-affinity binding site for Rev within RRE RNA is, in particular, reminiscent of the S8 binding site. In this case, the protein associates with a helical stem containing a five-base, purine-rich bulge (35) which is stabilized by a G·G, and possibly a G·A, base pair (36). The resulting distortion could provide access to functional groups within the major groove. According to genetic, footprinting and chemical-interference experiments, Rev binding entails a number of base-specific interactions with both the noncanonical base pairs as well as with adjacent, canonical base pairs (36−38). TAR RNA, to which the HIV Tat protein binds, consists of a helical stem interrupted by a three-nucleotide, U-rich bulge. Mutagenesis has shown that one of the bulged U residues and the two base pairs on the apical side of the bulge are involved in the interaction with Tat (39,40). A single arginine within the the basic domain of Tat appears to provide the only sequence-specific contact with the RNA (41). On the basis of a recent NMR study, it has been proposed that the arginine guanidinium group is hydrogen-bonded to the G of a G−C base pair adjacent to the bulge, as well as to two nearby phosphates, owing to stabilization by an unusual base triple formed by one of the bulged U residues and the A−U pair that stacks over the critical G−C pair (29,42). The extent to which the interaction between S8 and the 16S rRNA resembles those between Rev and RRE RNA, or Tat and TAR RNA, awaits a detailed structural analysis of the S8−rRNA complex. Nonetheless, the unusual configurations assumed by the RRE and TAR elements underscore the difficulties involved in predicting the tertiary structure of RNA segments that are not conventionally base paired.

Bulged bases and the autogenous regulation of ribosomal protein synthesis

Regulation of *spc* operon translation in *E.coli* is mediated by the association of protein S8 with a specific site in *spc* operon mRNA which is strikingly similar in both primary and secondary structure to the S8 binding site in 16S rRNA (9,19). The structural likeness between the two sites reinforces the notion that competition between rRNA and mRNA determines whether the protein enters the 30S subunit assembly pathway or serves as a feedback inhibitor of *spc* mRNA translation (43). By the same token, it can be concluded with some confidence that the same features of S8 take part in the two interactions. Indeed, we have found that a number of S8 mutants impaired to various extents in their rRNA-binding capacity exhibit a proportional decrease in their affinity for mRNA (H.Wu, I.Wower and R.A.Zimmermann, unpublished).

Despite the similarities in the rRNA and mRNA binding sites, the affinity of S8 for the former is approximately fivefold higher than for the latter. Assuming that this result can be extrapolated to the intact cell, we infer that S8 preferentially associates with 16S rRNA *in vivo* and that sigificant S8−mRNA interaction, leading to the inhibition of *spc* mRNA translation, occurs only when the availability of S8 exceeds that of 16S rRNA. We earlier proposed that the difference in binding constants for rRNA and mRNA might be attributable to the presence of two single bulged bases, U952 and C955, within a duplex portion of the mRNA site that corresponds to helix 21b in the rRNA site (9; see Figure 4). As shown here, the insertion of similar single-base bulges at equivalent positions within the rRNA binding site (rRNA15,16) reduced its affinity for S8 to a value typical of the mRNA site whereas deletion of the bulged base from the mRNA binding site (mRNA2,3,4) increased its affinity for S8 to a level similar to that of the rRNA (Figure 5). Single bulged bases are thus capable of modulating the strength of protein−RNA interactions, particularly when they occur in close proximity to essential recognition features.

The effects of single extrahelical nucleotides on the structure of B-form DNA have been studied in some detail by NMR and X-ray crystallography (e.g., refs. 44−48). Depending on the sequence context, environmental conditions and other molecular interactions, the unpaired base can either stack into the helix or protrude into the solution. While the stacked-in configuration does not disrupt adjacent base pairs, it leads to a kink in the helical stem. However, a base can be looped out without materially affecting either the pairing or the stacking of the surrounding nucleotides. Although the influence of single base bulges on the conformation of the A-form RNA helix has not been characterized with the same precision, NMR investigations have confirmed the presence of looped-out bases in duplex RNA structures, including one in which the bulged base is predicted to form an unusual

base triple with an adjacent base pair (30,49). Although the stacked-in configuration has not yet been conclusively demonstrated in RNA, there is no *a priori* reason why it cannot occur in RNA as well (see ref. 31). As the extrahelical bases in the mRNA binding site for S8 appear to antagonize S8–RNA interaction, they are not likely to be directly recognized by the protein. Rather, they make an unfavorable contact with the protein or cause local structural perturbations within the RNA that alter the conformation of the actual binding site. This role for bulged bases stands in contrast to that in the complex between bacteriophage R17 coat protein and R17 RNA in which a bulged A residue appears to be a key participant in the interaction (31). Thus, the presence of one or more bulged bases may reflect different functional ends. In the case of R17, the bulged base is essential for protein–RNA interaction, while in the interaction of S8 with *spc* operon mRNA, it may provide a mechanism for fine tuning the affinity between the RNA molecule and its protein ligand.

ACKNOWLEDGEMENTS

We are indebted to Dr Iwona Wower for helpful advice and discussion. This work was supported by grant DMB-9108104 from the National Science Foundation.

REFERENCES

1. Zimmermann, R.A. (1980) In Chambliss, G., Craven, G.R., Davies, J., Davis, K., Kahan, L. and Nomura, M. (eds.) Ribosomes: Structure, Function and Genetics. University Park Press, Baltimore, pp. 135–169.
2. Draper, D.E. (1989) *Trends Biochem. Sci.* **14**, 335–338.
3. Yates, J.L., Arfsten, A.E. and Nomura, M. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1837–1841.
4. Dean, D., Yates, J.L. and Nomura, M. (1980) *Nature* **289**, 89–91.
5. Müller, R., Garrett, R.A. and Noller, H.F. (1979) *J. Biol. Chem.* **254**, 3837–3878.
6. Zimmermann, R.A. and Singh-Bergmann, K. (1979) *Biochim. Biophys. Acta* **563**, 422–431.
7. Gregory, R.J., Zeller, M.L., Thurlow, D.L., Gourse, R.L., Stark, M.J.R., Dahlberg, A.E. and Zimmermann, R.A. (1984) *J. Mol. Biol.* **178**, 287–302.
8. Thurlow, D.L. (1981) Ph.D. Dissertation, University of Massachusetts, Amherst, Massachusetts.
9. Gregory, R.J., Cahill, P.B.F., Thurlow, D.L. and Zimmermann, R.A. (1988) *J. Mol. Biol.* **204**, 295–307.
10. Thurlow, D.L., Ehresmann, C. and Ehresmann, B. (1983) *Nucleic Acids Res.* **11**, 6787–6802.
11. Mougel, M., Eyermann, F., Westhof, E., Romby, P., Expert-BezanÇon, A., Ebel, J.-P., Ehresmann, B. and Ehresmann, C. (1987) *J. Mol. Biol.* **198**, 91–107.
12. Svensson, P., Changchien, L.-M., Craven, G.R. and Noller, H.F. (1988) *J. Mol. Biol.* **200**, 301–308.
13. Wower, I. and Brimacombe, R. (1983) *Nucleic Acids Res.* **11**, 1419–1437.
14. Ree, H.K., Cao, K. Thurlow, D.L. and Zimmermann, R.A. (1989) *Can. J. Microbiol.* **35**, 124–133.
15. Mougel, M., Allmang, C., Eyermann, F., Cachia, C., Ehresmann, B. and Ehresmann, C. (1993) *Eur. J. Biochem.* **215**, 787–792.
16. Gutell, R.R., Weiser, B., Woese, C.R. and Noller, H.F. (1985) *Prog. Nucleic Acid Res. and Mol. Biol.* **32**, 155–216.
17. Milligan, J.F. and Uhlenbeck, O.C. (1989) *Methods Enzymol.* **180**, 51–62.
18. Spierer, P., Bogdanov, A.A. and Zimmermann, R.A. (1978) *Biochemistry* **17**, 5394–5398.
19. Cerretti, D.P., Mattheakis, L.C., Kearney, K.R., Vu, L. and Nomura, M. (1988) *J. Mol. Biol.* **204**, 309–329.
20. Donis-Keller, H. (1979) *Nucleic Acids Res.* **7**, 179–192.
21. Webster, K.R., Shamoo, Y., Konigsberg, W. and Spicer, E.K. (1991) *BioTechniques* **11**, 658–661.
22. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Practical Approach, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
23. Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) *Methods Enzymol.* **185**, 60–89.
24. Wu, H., Wower, I. and Zimmermann, R.A. (1993) *Biochemistry* **32**, 4761–4768.
25. Laemmli, U.K. and Favre, M. (1973) *J. Mol. Biol.* **80**, 575–599.
26. Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254.
27. Brimacombe, R., Atmadja, J., Stiege, W. and Schüler, D. (1988) *J. Mol. Biol.* **199**, 115–136.
28. Mougel, M., Ehresmann, B. and Ehresmann, C. (1986) *Biochemistry* **25**, 2756–2765.
29. Puglisi, J.D., Chen, L., Frankel, A.D. and Williamson, J.R. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3680–3684.
30. Wimberly, B., Varani, G. and Tinoco, I., Jr. (1993) *Biochemistry* **32**, 1078–1087.
31. Wu, H.-N. and Uhlenbeck, O.C. (1987) *Biochemistry* **26**, 8221–8227.
32. Jessen, T.-H., Oubridge, C., Teo, C.H., Pritchard, C. and Nagai, K. (1991) *EMBO J.* **10**, 3447–3456.
33. Gait, M.J. and Karn, J. (1993) *Trends Biochem. Sci.* **14**, 335–338.
34. Jaffrey, S.R., Haile, D.J., Klausner, R.D. and Harford, J.B. (1993) *Nucleic Acids Res.* **21**, 4627–4631.
35. Heaphy, S., Finch, J.T., Gait, M.J., Karn, J. and Singh, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7366–7370.
36. Bartel, D.P., Zapp, M.M.L., Green, M.R. and Szostak, J.W. (1991) *Cell* **67**, 529–536.
37. Kjems, J., Calnan, B.J., Frankel, A,D. and Sharp, P.A. (1992) *EMBO J.* **11**, 1119–1129.
38. Tiley, L.S., Malim, M.H., Tewary, H.K., Stockley, P.G. and Cullen, B.R. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 758–762.
39. Churcher, M.J., Lamont, C., Hamy, F., Dingwall, C., Green, S.M., Lowe, A.D., Butler, J.G., Gait, M.J. and Karn, J. (1993) *J. Mol. Biol.* **230**, 90–110.
40. Hamy, F., Asseline, U., Grasby, J., Iwai, S., Pritchard, C., Slim, G., Butler, J.G., Karn, J., and Gait, M.J. (1993) *J. Mol. Biol.* **230**, 111–123.
41. Calnan, B.J., Tidor, B., Biancalana, S., Hudson, D. and Frankel, A.D. (1991) *Science* **252**, 1167–1171.
42. Puglisi, J.D., Tan, R., Calnan, B.J., Frankel, A.D. and Williamson, J.R. (1992) *Science* **257**, 76–80.
43. Nomura, M., Yates, J.L., Dean, D. and Post, L.E. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7084–7088.
44. Morden, K.M., Chu, Y.G., Martin, F.H. and Tinoco, I., Jr. (1983) *Biochemistry* **22**, 5557–5563.
45. Hare, D., Shapiro, L. and Patel, D.J. (1986) *Biochemistry* **25**, 7456–7464.
46. Kalnik, M.W., Norman, D.G., Zagorski, M.G., Swann, P.F. and Patel, D.J. (1989) *Biochemistry* **28**, 294–303.
47. Kalnik, M.W., Norman, D.G., Li, B.F., Swann, P.F. and Patel, D.J. (1990) *J. Biol. Chem.* **265**, 636–647.
48. Joshua-Tor, L., Frolow, F., Appella, E., Hope, H., Rabinovich, D. and Sussman, J.L. (1992) *J. Mol. Biol.* **225**, 397–431.
49. van den Hoogen, Y.T., van Beuzekom, A.A., de Vroom, E., van der Marel, G.A., van Boom, J.H. and Altona, C. (1988) *Nucleic Acids Res.* **16**, 5013–5030.
50. Gutell, R.R. (1993) *Nucleic Acids Res.* **21**, 3051–3054