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Role of conserved nucleotides in building the 16S rRNA binding site of *E.coli* ribosomal protein S8

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ABSTRACT

Ribosomal protein S8 specifically recognizes a helical and irregular region of 16S rRNA that is highly evolutionary constrained. Despite its restricted size, the precise conformation of this region remains a question of debate. Here, we used chemical probing to analyze the structural consequences of mutations in this RNA region. These data, combined with computer modelling and previously published data on protein binding were used to investigate the conformation of the RNA binding site. The experimental data confirm the model in which adenines A595, A640 and A642 bulge out in the deep groove. In addition to the already proposed non canonical U598 – U641 interaction, the structure is stabilized by stacking interactions (between A595 and A640) and an array of hydrogen bonds involving bases and the sugar phosphate backbone. Mutations that alter the ability to form these interdependent interactions result in a local destabilization or reorganization. The specificity of recognition by protein S8 is provided by the irregular and distorted backbone and the two bulged adenines 640 and 642 in the deep groove. The third adenine (A595) is not a direct recognition site but must adopt a bulged position. The U598 – U641 pair should not be directly in contact with the protein.

INTRODUCTION

The interaction of *E.coli* ribosomal protein S8 with its 16S rRNA binding site represents an interesting model for studying the molecular mechanism of specific RNA–protein recognition. Protein S8 is capable of binding individually to the central domain of 16S rRNA and plays an important role in the early stage of ribosomal 30S subunit assembly (1–2). It participates to the formation of one early nucleation site (3), and interacts co-operatively with other ribosomal proteins (4–5). It is therefore a crucial element for the sequential assembly of RNA and proteins constituting the small ribosomal subunit. It is also able to regulate the translation of its own operon (6–8) by a feed-back mechanism.

A considerable amount of work was already devoted to the interactions between S8 and its 16S rRNA target site and to the fine structure of this site (4–5, 9–14). It was recently shown that the rRNA can be restricted to a short helical stem (nucleotides 588–605/633–651), without significantly altering the apparent affinity constant (15). The central part of this helical region (called 'region C') is highly evolutionary constrained and the conserved elements are also found in the target regulatory site of S8 on its mRNA (8,16). We previously proposed a three-dimensional model of region C, derived from structure probing and computer modeling (14). This model displays characteristic features: A595, A640 and A642 bulge out in the deep groove of the helix, and U598 and U641 form a non-canonical base pair. However, the conformation of this region is disputed and three other folding models have been proposed in the literature. These models essentially differ in the pairing of U598 which is either with A640 (17–18), U641 (14) or A642 (5). We favoured a U595–U641 base pair (14), since it accounts for the non reactivity of U598 and U641 and for the reactivity of A640, A642 and A595. The pair U598–A640 was recently proposed on the basis of sequence comparison (17–18). In order to agree with the reactivity data, such a U598–A640 pair should involve Hoogsteen hydrogen bonding and not Watson–Crick interactions. In addition, the non reactivity of the unpaired U641 could only be explained by additional tertiary interaction or stacking.

Recently, we investigated the role of conserved nucleotides in region C as potential determinants for S8 recognition by studying the effect of 14 single and double mutations on S8 recognition (15). Of the 14 mutants tested, only three are still efficiently recognized by S8. In order to discriminate whether the loss of recognition is due to the loss of a specific contact or to conformational rearrangement, we now report the structural consequences of the mutations, using chemical probing on the 14 RNA variants mentioned above and of two new RNA mutants (A598/U640 and A598/U640/G641). In addition, footprinting experiments were conducted on those mutants that still retain S8 binding capacity. Our results emphasize the subtleties of RNA conformation and an unexpected versatility in the structural consequences of single base mutations. An improved three-

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dimensional model is derived from the present experimental data and the results are discussed in terms of RNA folding and S8 recognition.

MATERIALS AND METHODS

Preparation of the biological material

Plasmids construction, RNA synthesis and purification of wild-type and mutant 16S rRNA fragments (nucleotides 584–756) are described in (15). Two additional mutants were constructed (A598/U640 and A598/U640/G641) following the same protocol. Their relative binding affinity was determined as in (15). Ribosomal protein S8 was prepared under non-denaturing conditions according to Cachia *et al.* (19).

Chemical probing and footprinting

A standard assay contains 16 pmol RNA and 2 μg carrier tRNA in 20 μl of appropriate buffer. RNA was first pre-incubated for 15 min at 40°C in buffer N1 [50 mM sodium cacodylate (pH 7.5), 20 mM magnesium acetate, 250 mM potassium acetate] or N2 [50 mM sodium borate (pH 8.0); 20 mM magnesium acetate, 250 mM potassium acetate]. For each reaction, a control was treated in parallel, omitting the reagent. Modification with DMS: incubation was for 5 and 10 min in buffer N1 or for 2 and 5 min in buffer D1 [50 mM sodium cacodylate (pH 7.5), 1 mM EDTA] for semi-denaturing conditions. Modifications with CMCT: incubation was for 15 and 30 min in buffer N2 or for 2 and 5 min in buffer D2 [50 mM sodium borate (pH 8.0), 1 mM EDTA] for semi-denaturing conditions. Modifications with DEPC: incubation was for 15, 30 and 60 min in buffer N1 or for 15 and 30 min in buffer D1 (semi-denaturing conditions). All modifications were at 37°C. Footprinting experiments using CMCT and DMS were conducted on wild-type RNA and mutants allowing S8 binding. Complexes were formed in the presence of 0.4 μM S8 for wild-type RNA, mutants U595 and A641, or 2 μM for mutant A598–U640. Footprinting gels were scanned using the Bio-Imager Analyzer BAS 2000 (Fuji). Synthesis of primer, labeling, hybridization, reverse transcription and analysis of generated cDNA fragments were described by Mougel *et al.* (14).

Computer modeling

The modeled molecule integrating stereochemical constraints and experimental data was constructed with the help of several computer programs and tested by comparing the theoretical accessibility of atoms with the observed experimental reactivity, as described earlier (20).

RESULTS

Binding strength of the new mutants

Previous results showed that both mutants A598 and U642 fail to recognize S8 (15). Here, we tested the possibility to restore S8 binding by the double mutation A598/U642. The results (not shown) show that this double mutation restores only partially S8 recognition (with a 5-fold reduced binding strength). Sequence comparison indicates that U598 is highly conserved. However, in *Rcy purpur*, nucleotide 598 is an adenine, and nucleotides 640 and 641 are simultaneously replaced by U and G, respectively. Therefore, we constructed a new mutant containing these three mutations (A598/U640/G641). This triple mutant is not recognized by S8 (results not shown).

Conformational studies of the RNA variants

The four bases were tested for their chemical reactivity at one of their Watson–Crick positions with DMS, at A(N1) and C(N3), and with CMCT, at G(N1) and U(N3). For some mutants, position N7 of adenines was also probed with DEPC. In addition, footprinting experiments were conducted using DMS and CMCT with those RNAs that still retain S8 binding ability. A typical experiment is shown in Fig. 1. Experiments were repeated several times (from 2 to 4 times) and the degree of reactivity was evaluated from 1 to 4 by visual inspection. In the case of footprinting experiments, reactivity changes induced by S8 binding were quantified.

The reactivity changes induced by the mutations are exclusively localized in region C (nucleotides 594–599/639–645). Results are summarized in Table 1 and in Figs 2–4 which show the deduced secondary foldings of region C. One striking consequence of all the mutations tested is that U641, which is not reactive in the wild-type RNA, becomes reactive at various degrees in all mutated RNAs, with the single exception of mutant G643 (Table 1). By contrast, U598 remains unreactive in all mutants, suggesting that its N3 position is involved in H-bonding or that the residue is stacked inside the helix, preventing modification.

DISCUSSION

Mutations affecting adenines 595, 640 and 642

The deletion of any of these three adenines results in a complete loss of binding (15). The deletion of either A640 or A642 induces reactivity at U641 and decreases the reactivity of A642(N1) or A640(N1), respectively (Table 1). These results suggest that nucleotide U641 is bulging out in these two mutants and that U598 pairs with either A642 or A640, respectively (Fig. 2). Moreover,

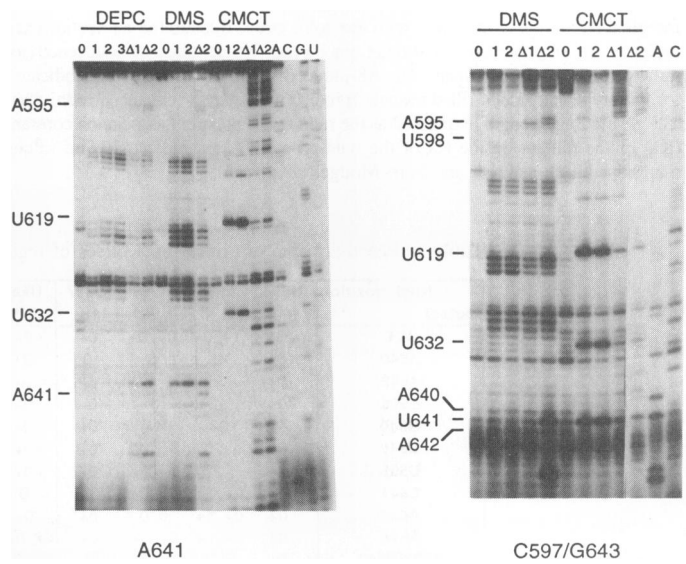


Figure 1. Probing on RNAs A641 and C597/G643. Modification with DMS and CMCT: Incubation control (lane 0). Native conditions: incubation with DMS or CMCT was for 5 min (lane 1) and 10 min (lane 2); semi-denaturing conditions: incubation with CMCT was for 5 min (lane Δ1) and 10 min (lane Δ2). Modification with DEPC: incubation control (lane 0); native conditions: incubation for 15 min (lane 1) and 30 min (lane 2); semi-denaturing conditions: incubation for 5 min (lane Δ1) and 10 min (lane Δ2).

the reactivity of A595(N1) increases by 2-fold, indicating that A595 is not simply bulged out in the wild-type RNA (as already hinted at by its low reactivity) but is probably involved in hydrogen-bonding or stacking, interactions which are disrupted in each deletion mutant. Thus, the observed lack of binding of protein S8 may be due to the loss of a possible contact and/or to a local structural rearrangement of region C. Unexpectedly, the deletion of A595 induces a high level of reactivity at U641 (level 3), and a 2-fold increase in the reactivity of A642 (Fig. 2), showing that the removal of the bulged A595 destabilizes the interactions which involve U641. The non-reactivity of U598 suggests that it remains stacked inside the helix, either unpaired or alternatively paired with A640 or A642.

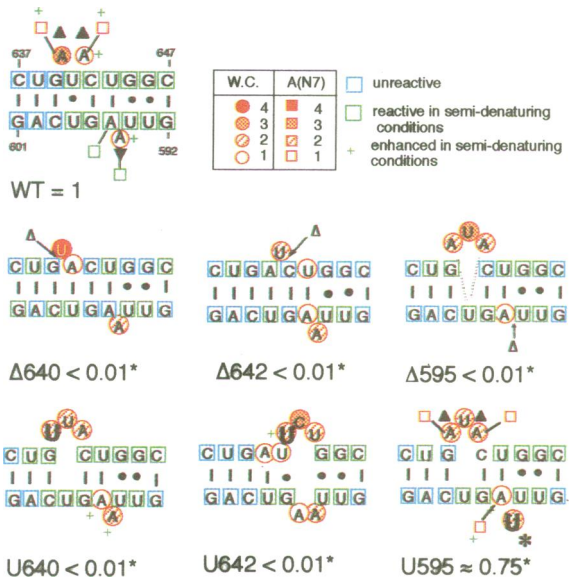


Figure 2. Proposed secondary structure of mutants affecting adenines 595, 640 and 642. The wild-type RNA is shown as reference. Only nucleotides 592–601/637–647 are shown. The reactivities (estimated between 1 and 4 from marginal to high) are indicated with the color code. Substituted nucleotides are indicated in bold characters and deletions by (Δ). Nucleotides without reactivity indicated are not determined. The S8-induced reactivity changes are indicated for wild-type and U595: filled triangle (protection); asterisk (enhanced reactivity). The S8 binding strength [expressed as the ratio of the apparent association constant (Ka) of the mutant on the Ka of the wild-type RNA]are indicated. The values marked with an asterisk are from Mougel *et al.* (15).

The A to U substitution at position 642 causes the disruption of the G597–C643 pair since C643 becomes highly reactive at N3 (level 3). The reactivity pattern favors the existence of two base pairs, U598–A640 and G597–U641, while nucleotides A595, A596, U642, C643 and U644 form an interior asymmetric loop (Fig. 2). Thus, the loss of binding induced by the U642 mutation results from a refolding of region C. In mutant U640, U641 becomes reactive (level 2) but less than in mutant ΔA595 (level 3). Therefore, the interaction involving U641 might be weakened but not completely abolished. Another consequence of the A640 substitution is the 2-fold increase in reactivity of A595(N1), as already observed in mutants Δ640 and Δ642. Since the deletion of A595 has also a distal effect on U641 and A642, a structural interdependence between A595, U598, A640 and A642 can be inferred.

Mutant U595 requires a particular attention since it is still recognized by S8 with the same affinity as the wild-type RNA (15). Its reactivity pattern is rather similar to that of mutant U640 (Table 1). However, U641 becomes reactive (level 2), revealing an unexpected distal effect induced by the mutation. The fact that mutant U595, but not mutant U640, is recognized by S8 suggests that A640 is a specific determinant for S8, and that a bulged nucleotide, but not necessarily an adenine, is required at position 595. Most likely, this bulged nucleotide or the particular distortion of the backbone induced by this bulge, is necessary for a correct RNA fold. Since both U595 and U641 are reactive in this mutant (level 2), it was interesting to test their reactivity in the S8–RNA complex. The footprinting experiments show that A640 and A642 become unreactive as in the case of the wild-type RNA. However, U641 displays the same level of reactivity as in the naked RNA and the reactivity of U595 is even increased by a factor of 2 (not shown). This observation confirms that nucleotide 595 is not a specific contact but is required as a bulge. Note that U641 remains unreactive in the wild-type RNA–S8 complex.

Possible interactions involving U598

In the different models proposed so far, U598 is paired with either A640, U641 or A642. The U598–U641 pair was tested by

Table 1. Reactivity data of critical nucleotides of region C in wild-type and mutant RNAs

Nucl. position / Mutant	U594 (N3)	A595 (N1,N7)	A596 (N1,N7)	G597 (N1)	U598 (N3)	C599 (N3)	G639 (N1)	A640 (N1,N7)	U641 (N3)	A642 (N1,N7)	C643 (N3)	U644 (N3)	G645 (N1)
WT	0+	1+, 0+	0+, 0+	0+	0+	0	0	2, 1+	0+	1+, 1+	0+	0+	0+
Δ640	0+	2	0	0	0+	0+	0	Δ	4	1	0	0+	0
Δ642	0+	2	1	0	0+	0	0	0+	2	Δ	0	1	0
Δ595	0+	Δ	1	0+	0+	0	0+	2	3	2	0+	0+	0+
U640	0+	2+	1+	0+	0+	0+	0+	2	2	2	0+	0+	0+
U642	0+	2	1	0+	0+	0	0	1	1+	2	3	2	0+
U595	0+	2	1+,1+	0+	0+	0	0	2, 1	2	2, 1	nd	0+	0+
C641	0+	2+, 2+	1+, 0+	0	0	0	0	5, 0	nd	1+, 1+	0+	1	0+
A641	0+	0+, 1+	0, 0	0+	0+	0	0+	3,1+	0+, 0	2, 1	0+	0+	0+
A598	0+	0+, 2+	0+, 0+	0+	2, 0+	0	0	2, 2	2	2, 2	0	0+	0+
A598/U642	0+	1+, 3	0+,0+	0	2, 0+	0+	0+	2,2	4	3	0	0+	0+
G643	1	4	2	0+	0+	0	0	0	0+	2	nd	3	0+
C597/G643	1+	2+	1	0+	0+	0	0	2	3	2	0	0+	0+
G599	0+	1+,4	0	0+	0+	0+	1+	3,3	2	2,2+	0	0+	0+
G599/C639	0+	1+	0+	0+	0+	0	0	2	1+	2+	0	0+	0+
A598/U640	0+	1+	1+	0	2	nd	0	0	1+	2	1	0+	0+
A598/U640/G641	0	0, 1+	0, 1+	0	0+, 1+	0	0	1, 3	0	3, 3	0+	0+	0+

The degree of reactivity of U(N3), G(N1), C(N3), A(N1) (first number) and A(N7) (second number) is estimated from 1 to 4, as in Fig. 3 and 7–9. Degree 5 corresponds to an hyperreactivity (enboxed). Reactivity of mutated nucleotides is shadowed. (+) denotes reactivity or increase of reactivity in semi-denaturing conditions; (nd) is not determined.

substituting U641 either by C (preventing the formation of the U−U pair but not the pairing between U598 with either A640 or A642), or by A (allowing the formation of a potential canonical U−A pair). Unexpectedly, the C641 mutation results in hyperactivity of A640(N1) (with N7 unreactive), while U598 remains unreactive under native conditions (Table 1). Unfortunately, a pause of reverse transcriptase masks the mutated C641. Otherwise, the reactivity of A595 is enhanced 2-fold at both N1 and N7. Although we have no obvious explanation for the hyperactivity of A640, this results precludes the formation of a canonical U598−A640 pair in this mutant (but not a Hoogsteen pair). On the other hand, in mutant A641 the mutated adenine is unreactive at both N1 and N7, while A640 and A642 are reactive (with A640 > A642) (Fig. 1). This result indicates that U598 does form a canonical pair with the mutated A641 but not with A640 or A642, although the three adenines are potential candidates for pairing (Fig. 3). Furthermore, this mutant is perfectly recognized by S8. Moreover, A640 and A642 are clearly protected from DMS modification in the presence of S8 (not shown). On the other hand, the substitution of U598 by A leads to a loss of S8 binding (15). Interestingly, probing indicates that A598 and U641 do not form a stable inverted pair, as shown by the reactivity of these two nucleotides (level 2) (Fig. 3). It is puzzling that the U598−A641 pair can be formed, while the inverted A598−U641 cannot. This results supports the existence of an unusual U598−U641 pair, however.

The other two alternatives imply the formation of a base pair between U598 with either A640 or A642. We first showed that substituting A640 or A642 by U leads to a loss of binding and induces local rearrangements (see below). However, the double A598/U642 substitution does not restore binding (15). In fact, in this double mutant, U641 and U642 are both highly reactive (with U641 > U642), indicating that the mutated A598 which is also reactive (level 2) does not pair with any of the two potential candidates (U641 or U642). Most likely, residues 598 and 640−642 are unpaired and form an interior open loop (Fig. 3). Furthermore, the reactivity of A595 remains unchanged at N1 but becomes reactive at N7 (level 3). In this study, we showed that the double A598/U640 substitution restores only partially S8 recognition. Probing experiments indicate that A598 is reactive (level 2) and U640 unreactive. Strikingly, U641 is only marginally reactive (2-fold less than in RNA A598), but becomes more reactive in semi-denaturing conditions. Thus, there is no clear evidence for a pairing of A598 with either U640 or U641 (Fig. 3). We also showed that the triple mutant A598/U640/G641 is not recognized by S8. Probing experiments indicate that only A642 is highly reactive at both N1 and N7 (level 3). Again, it is not clear from probing data whether A598 interacts with U640 or G641.

Our results also points out the limits of nucleotide sequence comparison while ignoring amino acid sequence co-variations in the corresponding protein. Indeed, a phylogenetic analysis of a subset of protein L23/25 and their putative respective rRNA binding sites clearly evidenced the existence of co-variations in both RNA and protein (21). Therefore, none of the postulated pairs involving U598 can be strictly proven by the classical disruption/inversion method. The only positive mutant (A641)

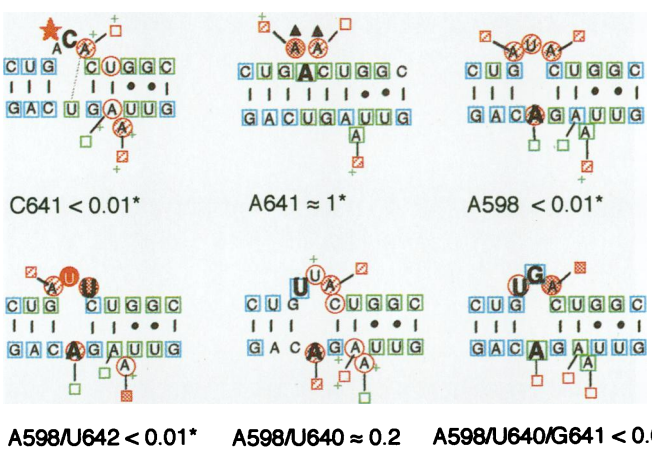


Figure 3. Proposed secondary structure of mutants affecting the possible interactions involving U598. Same legend as in Fig. 2. The red star denotes an hyperreactivity.

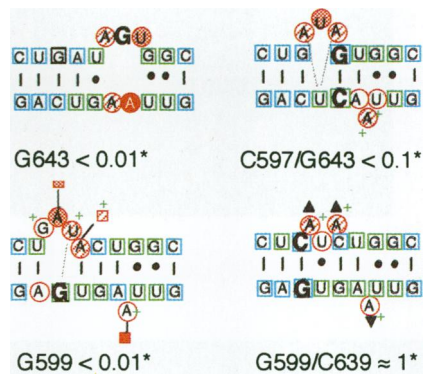


Figure 4. Proposed secondary structure of mutants affecting the G−C pairs. Same legend as in Fig. 2. The S8-induced protections from DMS are indicated for mutant G599/C639 (in the presence of 2 μM of S8).

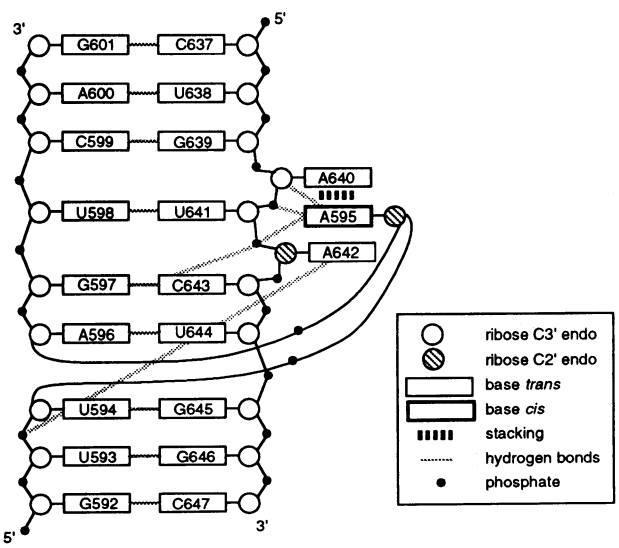
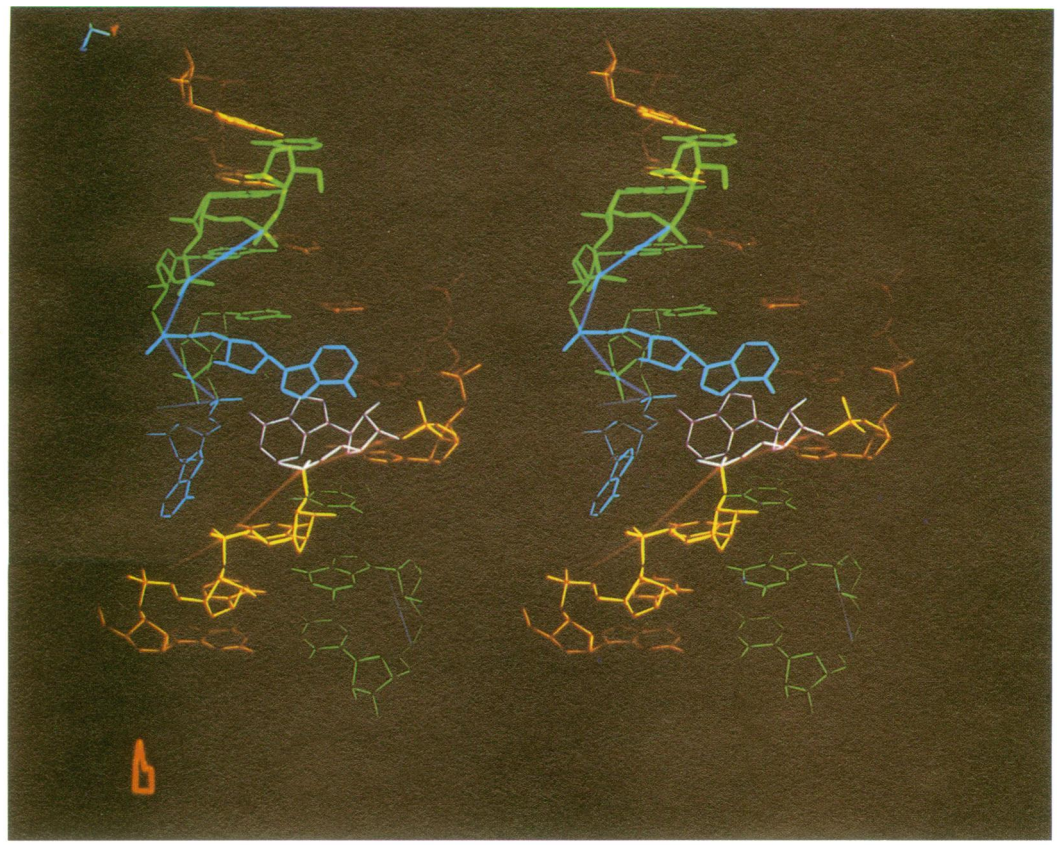
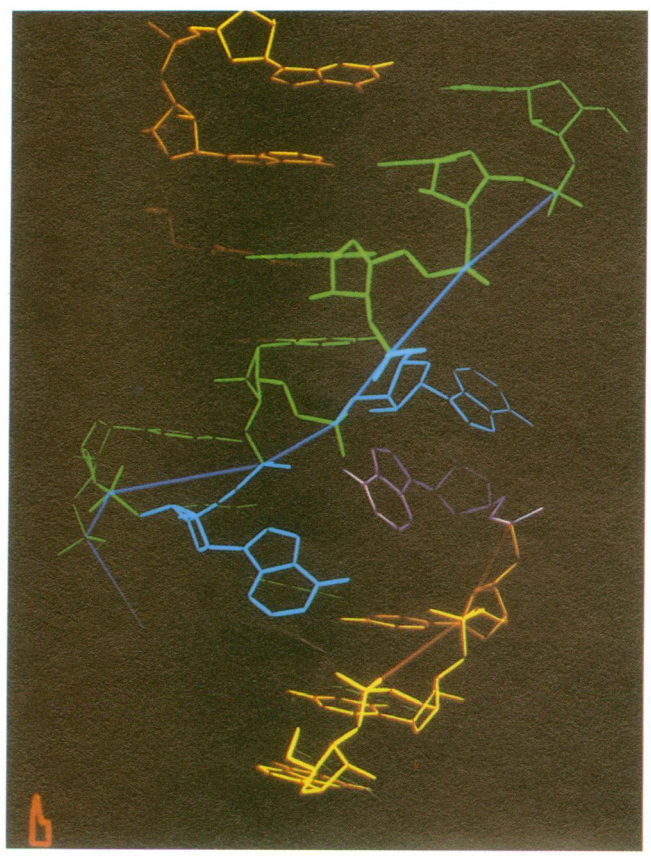


Figure 5. Schematic diagram of the proposed conformation of region C.

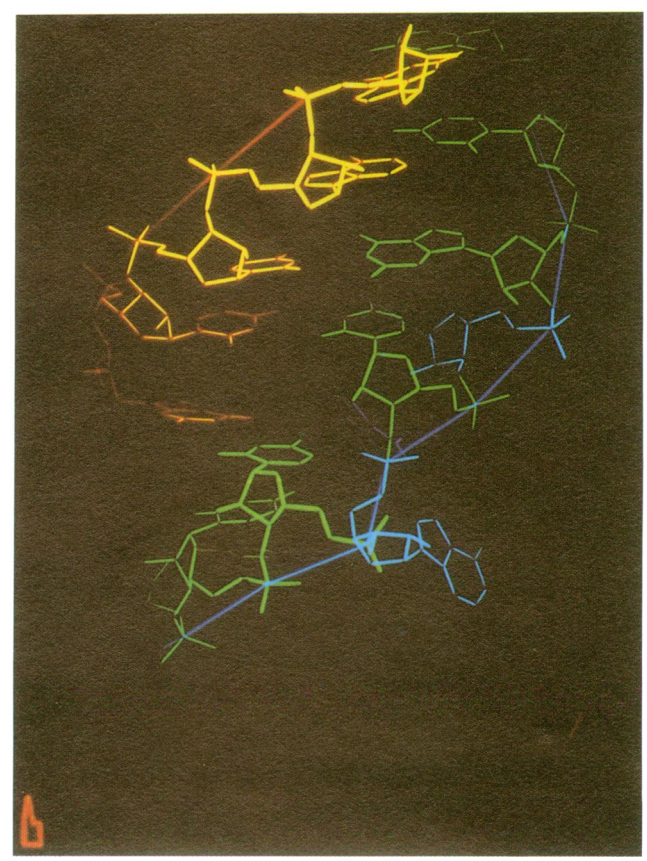
a



b



c



favors the existence of the U598–U641 pair, since the formation of a U598–A641 pair was clearly evidenced. One should note that U641 is frequently substituted by A in 16S-like rRNAs.

Mutations affecting the G–C pairs

From our model, the G599 and G643 substitutions, which both lead to a complete loss of S8 binding (15), are expected to disrupt the two G–C pairs surrounding the U–U pair and most likely to have a destabilizing effect. Indeed, reactivity data indicate that these mutations induce conformational rearrangements. As a result of the G643 mutation, A640(N1) becomes unreactive while the reactivity of A595(N1) is enhanced by a factor of 4 (Table 1). In addition, U644 and A596(N1) become reactive (level 3 and 2, respectively). These data suggest the formation of pairs U598–A640 and G597–U641 (as in mutant U642). Unexpectedly, the potential A595–U644 pair is not formed and nucleotides 595, 596 and 642–644 form a five-base internal loop (Fig. 4). The G599 mutation induces a 2-fold increase of reactivity at A640 (both N1 and N7 positions). It also induces new reactivity at U641 (level 2) and at A595(N7) (level 4). Most likely, helix III is extended by the two meta-stable U598–A642 and G599–U641 pairs (Fig. 4).

The double mutation G599–C639 restores S8 binding (15) and gives a reactivity pattern similar to that of the wild-type RNA (Table 1). The only difference with the wild-type RNA is a marginal reactivity of U641 (level 1) in native conditions, and a 2-fold increase of the reactivity of A640(N1). Therefore, the C599–G639 base pair can be inverted without significant functional and structural effect (Fig. 4). On the contrary, the double mutation C597/G643 does not restore binding (15). Probing experiments show that the mutations cause a strong reactivity of U641 (level 3) and a 2-fold increased reactivity of A595 and A640 at N1 (Fig. 1). The high reactivity at U641 could be explained by the possible loss of interactions with U598 as a consequence of the mutations or to the alteration of a network of interactions involving other nucleotides like A595. Thus, an inverted C597/G643 pair is formed but it is not structurally equivalent to the wild-type one. However, specific contacts between S8 and this G–C pair cannot be excluded. Interestingly, the G597–C643 pair is strictly conserved. Note that a C to U transition and a single deletion at position 643, both produce over 50-fold reduction in S8 affinity and confer slow growth in *E.coli* cells *in vivo* (16).

A possible three-dimensional model

The present results show that the fold of region C is functionally and structurally highly constrained. The effect of mutations could not be predicted by a simple secondary structure model. The mutations can be classified in 3 classes: (i) mutations that display a wild-type like folding and affinity for protein S8 (A641 and G599/C639); (ii) mutations that induce a substantial refolding (Δ640, Δ642, U642, C641, G643 and G599) and are not recognized by protein S8; (iii) mutations that induce a local opening of region C (Δ595, U595, U640, A598, A598/U642, A598/U640, A598/U640/G641 and C597/G643) with variable effect on S8 binding. These latter mutations seem to be responsible for the disruption of a network of interactions in region C resulting in a destabilization of the postulated U598–U641 pair. Furthermore, there is a clear structural interdependence between nucleotides A595, U598, A640, A642 and G597 and/or C643. The new model we propose does not basically differ from the previous one, as far as the base-pairing scheme is concerned, however the conformation of the sugar-phosphate backbone is more irregular and tertiary interactions account for the present observations (Figs 5–6).

In this model, the U598(N3, O4)–U641(O2, N3) already proposed in the previous model (14) has been maintained. The three bulged adenines are still bulging out on the same side of the helix, facing the major groove, but their orientation has been modified. Both A595 and A640 adopt a C2′ *endo* sugar pucker. Adenine 595, which is in a *syn* conformation, is stacked on A640 and both residues can be involved in an array of hydrogen bonds (Figs 5–6). Thus, hydrogen bonding between A595(N6) and the phosphate groups of both U641 and A642, between A595(N7) and the 2′OH of A640, as well as between A642(N6) and the phosphate group of U594 can occur. There is a very good correlation between the reactivity of A640 and A595 at both N1 and N7 and their accessibility in the model. Moreover, the postulated hydrogen bonds involving A595 and the ribose-phosphate backbone most likely stabilize its particular conformation. Thus, according to the model, deleting or substituting A595 results in the loss of these interactions and to the destabilization of the U598–U641 pair. The free hydrogen of C643(N4) can also form a bond with the phosphate group of A642. This should account for the observed increased reactivity of both A642 and U641 when inverting the G597–C643 pair. This model offers a rather satisfying solution for the observed interdependence between the three bulged adenines, the U–U pair and C643. Other hydrogen bonding possibilities cannot be excluded. Overall, the postulated structure is characterized by: (i) the known tendency of R-Y-R sequences for conformations in which the two purine residues stack on a side opposite to that of the pyrimidine (22); (ii) the added stabilization brought about by the third adenine 'intercalating' between the two bulged adenines.

What is recognized by protein S8?

One characteristic feature of the model is the irregularity of the sugar-phosphate backbone (with one kink on the 5′ strand and two kinks on 3′ strand). The reason why the U598–U641 pair can be replaced by U598–A641 but not by A598–U641 is probably correlated with this particular geometry. Another consequence of the proposed conformation is the widening of the deep groove, allowing to position the three bulged adenines. Protein S8 may sit in the distorted deep groove of the RNA and probably recognizes the irregular backbone conformation. The model also fits with the idea that A640(N1), which is accessible in the naked RNA and protected in the bound form, is a specific contact. The invariant A642 is also a good candidate for specific interaction, in particular positions N6 and N1 which are both accessible in the model. It should be reminded that S8 binding is strongly affected by protonation of (a) residue(s) with a pK around 5–6 (13) and that an adenine was considered to be the best candidate. On the opposite, A595 which is buried and poorly

Figure 6. Proposed three-dimensional model of region C. (**a**) Stereoscopic view down the deep groove, with strand 637–647 in green, strand 592–601 in yellow, A595 in pink, A640 and A642 in blue. (**b**) Detailed view showing the coaxial stacking between A640 and A595 and possible hydrogen bonds (A595(N6)–OP641 and –OP642; A595(N7)–A640(2′OH); A642(N6)–OP594). (**c**) Detailed view after a rotation of 180° about the vertical axis, showing the U598–U641 and the possible hydrogen bond between C643(N4) and OP642.

accessible does not appear to be involved in direct interaction. The evidence that A595 is not a recognition site is provided by mutant U595. In this case, the reactivity of U595(N3) is even increased in the presence of S8, suggesting that it is tilted outside the helix. The fact that U641(N3) remains reactive in this mutant in the presence of S8 also indicates that U641 is probably not directly recognized. This can be explained by the particular location of the U598–U641 pair: in the proposed model, its access from the distorted deep groove is partially shielded by the bulged adenines.

The S8 binding site constitutes a typical example of RNA structural complexity used as a source of protein specific recognition. Our results highlight subtleties in the RNA conformation which cannot be explained by a simple secondary structure. In addition, they clearly show that the classical disruption/replacement method used to prove standard Watson–Crick base-pairing is inadequate for identifying non canonical interactions.

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