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The phylogenetically conserved doublet tertiary interaction in domain III of the large subunit rRNA is crucial for ribosomal protein binding

(protein–RNA interaction/yeast/*Saccharomyces cerevisiae*/evolution)

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ABSTRACT **Previous phylogenetic analysis of rRNA sequences for covariant base changes has identified ≈20 potential tertiary interactions. One of these is present in domain III of the large subunit rRNA and consists of two adjacent Watson–Crick base pairs that, in *Saccharomyces cerevisiae* 26S rRNA, connect positions 1523 and 1524 to positions 1611 and 1612. This interaction would strongly affect the structure of an evolutionarily highly conserved region that acts as the binding site for the early-assembling ribosomal proteins L25 and EL23 of *S. cerevisiae* and *Escherichia coli*, respectively. To assess the functional importance of this tertiary interaction, we determined the ability of synthetically prepared *S. cerevisiae* ribosomal protein L25 to associate *in vitro* with synthetic 26S rRNA fragments containing sequence variations at positions 1523 and 1524 and/or positions 1611 and 1612. Mutations that prevent the formation of both base pairs abolished L25 binding completely, whereas the introduction of compensatory mutations fully restored protein binding. Disruption of only the U^{1524}·A^{1611} pair reduced L25 binding to ≈30% of the value shown by the wild-type 26S rRNA fragment, whereas disruption of the G^{1523}·C^{1612} base pair resulted in almost complete loss of protein binding. These results strongly support the existence and functional importance of the proposed doublet tertiary interaction in domain III of the large subunit rRNA.**

Ribosomes are highly complex ribonucleoprotein particles that catalyze the sequential linkage of amino acids in the order specified by the codon sequence of each particular mRNA. Initially, the biological activity of the ribosome was thought to reside in the protein moiety. Over the past years, however, a considerable body of evidence has been collected in support of the view (1) that the rRNAs, rather than merely ensuring the correct spatial arrangement of the ribosomal proteins, participate directly in ribosomal function (for a recent review, see ref. 2) and might even be the fundamental functional determinants of the ribosome (3, 4). Regions crucial for each of the three main phases of translation have been identified in both the small and large subunit rRNA, predominantly by *in vivo* mutational analysis of the *Escherichia coli* rRNA species and by studies on the nature of various antibiotic-resistant mutations (for reviews, see refs. 2, 5, and 6). The strong evolutionary conservation of the primary and/or secondary structure of most of these regions supports their importance for the pertinent biological functions in all types of ribosomes.

Phylogenetic comparison of the large number of rRNA sequences presently available has provided us with a fairly detailed picture of the secondary structure of the different types of rRNA (7, 8). Our knowledge of the three-dimensional folding of these molecules, which is of paramount importance for a full understanding of their function, is still very limited, however. Relatively rough models of the conformation of *E. coli* 16S and 23S rRNA have been developed (9, 10), but these still require substantial refinement. One way to approach this goal is by identification of tertiary interactions between nucleotides. A number of potential tertiary interactions have been uncovered by the same type of analysis used in developing the secondary structure models, i.e., by searching the data base of rRNA sequences for covariant base changes (11, 12). So far, however, only 2 of the ≈20 proposed tertiary interactions have been experimentally confirmed, again by mutational analysis of the *E. coli* rRNA species. Both occur in regions that are among the most highly conserved functional elements in the rRNA: one in the "530 stem–loop" of the small subunit and the other in the "GTPase center" of the large subunit rRNA. In the first case, perturbation of the proposed Watson–Crick base pairing between residues 524–526 and 505–507 of 16S rRNA proved to have a severe negative effect upon growth of *E. coli* cells containing predominantly mutant rRNA (13). In the second case, a synthetically prepared fragment of *E. coli* 23S rRNA carrying a disruption of the proposed U^{1082}·A^{1086} base pair was found to have suffered at least a 10-fold reduction in affinity for the ribosomal protein EL11† as well as the antibiotic thiostrepton (15), which bind directly to the GTPase center (6, 16).

We have been interested in another highly conserved functional region, located in domain III of the large subunit rRNA, that acts as the binding site for the early-assembling ribosomal proteins EL23 in *E. coli* and L25 in *Saccharomyces cerevisiae* (17, 18). Previous experiments showed that both ribosomal proteins, despite very limited sequence similarity (19), recognize both the homologous and heterologous large subunit rRNA with similar specificity and efficiency (18, 20). Phylogenetic analysis of this conserved ribosomal protein binding site (11, 12) has revealed a possible tertiary interaction between residues 1343 and 1344 and residues 1403 and 1404 in *E. coli* (or between residues 1523 and 1524 and residues 1611 and 1612 in *S. cerevisiae*; see Fig. 2). As part of our efforts to define the molecular details of the evolutionarily conserved binding of ribosomal protein to this region of the large subunit rRNA, we have verified this tertiary interaction by testing the effect of disruption and restoration of the proposed base pairing upon the specific *in vitro* binding of L25 to a synthetic yeast 26S rRNA fragment.

MATERIALS AND METHODS

Mutagenesis. Mutations were introduced into the L25 binding site by first cloning the 2.5-kilobase *Xba* I–*Hin*dIII

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†*E. coli* r-proteins are designated by the suffix E; yeast r-proteins are designated by the nomenclature of Kruiswijk and Planta (14).

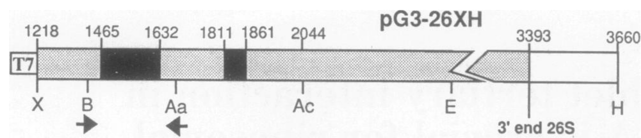

FIG. 1. Restriction map of the yeast rRNA gene insert of plasmid pG3-26XH. The region encoding part of the 26S rRNA sequence is shaded, and the bipartite L25 binding region (cf. Fig. 2) is solid. T7 indicates the bacteriophage T7 promoter sequence. Numbering is relative to the 5′ end of the mature 26S rRNA sequence. The arrows indicate the primers used for PCR mutagenesis. Aa, *Aat* II; Ac, *Acc* I; B, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; X, *Xba* I.

rRNA gene fragment (Fig. 1) from pG3-26XH (21) into the pSELECT vector (Promega). Mutagenesis was then carried out by the supplier's instructions. Alternatively, mutagenesis was performed by the polymerase chain reaction (PCR) as described by Perrin and Gilliland (22). In this case, a derivative of pG3-26XH was used in which the *Aat* II site present in the vector sequence had been destroyed. The 5′ and 3′ PCR primers were chosen to overlap the *Bgl* II and *Aat* II sites in the rRNA gene insert, respectively (cf. Fig. 1). Synthetic deoxyoligonucleotides were synthesized in our laboratory on an Applied Biosystems 381A DNA synthesizer. Appropriate mutants were selected by double-stranded dideoxynucleotide sequencing (23). Subsequently, either the *Bgl* II–*Eco*RI or *Bgl* II–*Aat* II mutant rRNA gene fragment (cf. Fig. 1) was isolated and used to replace its wild-type counterpart in pG3-26XH resulting in the pG3-26XHm series of plasmids.

***In Vitro* Transcription and Translation.** Synthetic biotinylated 26S rRNA fragments encompassing the complete wild-type L25 binding site or mutant versions thereof were prepared by linearizing plasmid pG3-26XH or individual members of the pG3-26XHm series with *Acc* I followed by *in vitro* run-off transcription with T7 RNA polymerase in the presence of bio-11-UTP (Sigma) as described (21). [^{35}S]Methionine-labeled ribosomal protein L25 was obtained by *in vitro* run-off transcription of linearized plasmid pG4-L25, which contains the complete L25 cDNA under control of the phage T7 promoter, followed by *in vitro* translation of the transcript in a wheat germ translation system (Promega) containing [^{35}S]methionine at 15 μCi/μl (1500 Ci/mmol; 1 Ci = 37 GBq; Amersham) as described (21).

Binding Assay. Binding of L25 to the synthetic 26S rRNA fragments was assayed by incubating the ^{35}S-labeled synthetic protein with a 100- to 500-fold excess of biotinylated rRNA fragment under the appropriate conditions and precipitating the rRNA-bound protein with the aid of avidin-agarose (Pierce) as described (21). The amount of bound protein was then quantitated by gel electrophoretic analysis or by precipitation with trichloroacetic acid of the material attached to the agarose beads (21). As a control, the protein was incubated with a *S. cerevisiae* 17S rRNA fragment encompassing nucleotides 1–580.

RESULTS AND DISCUSSION

Effect of Disruption of the Pairing of Positions 1523 and 1524 with Positions 1611 and 1612 on Binding of L25. Fig. 2 depicts the secondary structure of part of domain III of *S. cerevisiae* 26S rRNA, which we have shown to be specifically recognized by yeast ribosomal protein L25 and by the homologous ribosomal protein EL23 from *E. coli*, that normally binds to the corresponding region in the bacterial 23S rRNA (20). Nucleotides 1523 and 1524 and nucleotides 1611 and 1612, shown in reversed contrast, are thought to be involved in tertiary folding of this region because of their evolutionarily conserved potential to form two adjacent Watson–Crick base pairs (11, 12).

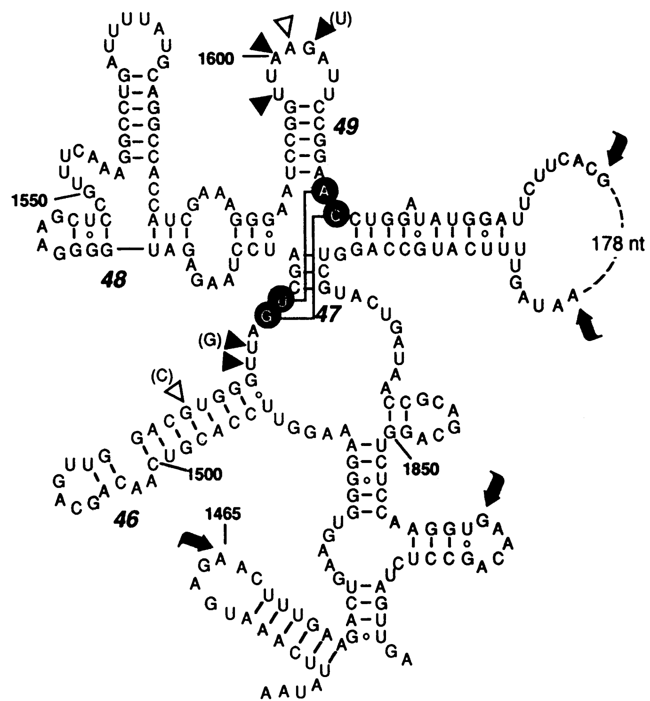

FIG. 2. Secondary structure of the bipartite L25 binding region in domain III of *S. cerevisiae* 26S rRNA. Numbering of the residues is relative to the 5′ end of the 26S sequence. The arrows indicate the limits of the protection of the rRNA by L25 against RNase T1 digestion (18). Nucleotides involved in the tertiary interaction are shown in reversed contrast. Numbering of the helices is according to the scheme used in ref. 8. Triangles point to residues that are weakly (open) or strongly (solid) protected against chemical modification in the corresponding region of *E. coli* 23S rRNA by binding of the *E. coli* EL23 homolog of L25. Where the nature of the protected residue differs between the two organisms the *E. coli* residue is shown in parentheses.

To verify this tertiary interaction experimentally, we tested the ability of L25 to bind *in vitro* to two sequence variants of the region in question. (*i*) The potential base pairing between nucleotides G^{1523}-U^{1524} and A^{1611}-C^{1612} was completely disrupted by changing the former sequence to AC (Fig. 3*A*, mutant I). (*ii*) The potential to interact was restored by introduction of an additional double mutation changing A^{1611}-C^{1612} to GU (Fig. 3*A*, mutant II). Binding of L25 to the wild type and each of the mutant sequences was analyzed by an *in vitro* assay (21) in which [^{35}S]methionine-labeled synthetic L25 was incubated with a 100- to 500-fold excess of an *in vitro*-transcribed 26S rRNA fragment encompassing the complete L25 binding site and labeled with biotinylated UTP. The biotinylated RNA, together with any bound protein, was then removed from the incubation mixture with the aid of avidin-agarose beads and the amount of labeled protein attached to the beads was analyzed either by SDS/gel electrophoresis followed by autoradiography or by precipitation with trichloroacetic acid. Fig. 3*B* shows the autoradiograph from a representative experiment. Clearly, mutant I completely lost the ability to be recognized specifically by L25. The amount of protein removed from the reaction mixture by avidin-agarose beads after incubation with the mutant 26S rRNA fragment did not exceed the background level obtained upon incubation with a synthetic 17S rRNA fragment of similar length (cf. Fig. 3*B*, lanes I and C). L25 binding was restored to virtually wild-type level, however, upon introduction of the compensating mutations at positions 1611 and 1612 in mutant II (cf. Fig. 3*B*, lanes II and W). Quantification of the amount of labeled L25 present in the RNA–protein complex, either by scanning of the autoradiograph or trichloroacetic

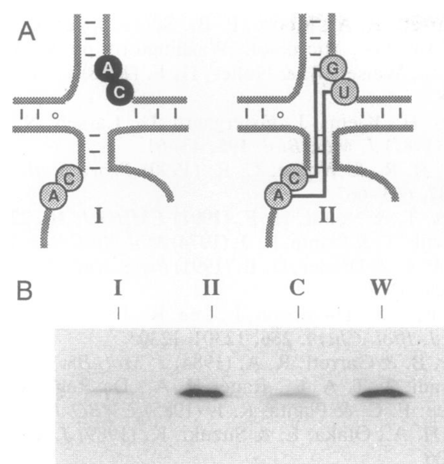

FIG. 3. Effect of disruption and restoration of the tertiary interaction on binding of ribosomal protein L25. (A) Schematic representation of the structure of the mutants used. Mutated nucleotides are shaded. Possible pairing is indicated by lines connecting the residues involved in the tertiary interaction. (B) Gel electrophoretic analysis of the ^{35}S-labeled material removed from the reaction mixture by the avidin-agarose beads after incubation of the [^{35}S]methionine-labeled synthetic L25 with biotinylated synthetic wild-type 26S rRNA fragment (lane W), mutant fragments I and II (lanes I and II, respectively), or a biotinylated synthetic 17S rRNA fragment of similar length (lane C).

acid precipitation (see Table 1), showed the efficiency of binding of mutant II to be indistinguishable from that observed with the wild-type 26S rRNA fragment. This result provides strong support for the actual existence of the proposed tertiary interaction in domain III of the large subunit rRNA.

Effect of Disruption of Individual Base Pairs. Although phylogenetic analysis supports the existence of a doublet tertiary interaction in domain III of the large subunit rRNA, the evidence for the pairing between positions 1523 and 1612 is relatively weak. So far only a few cases of covariance in this pair have been observed, which are limited to the eukaryotic kingdom (12). We, therefore, analyzed the binding of L25 to two additional sequence variants of domain III containing either a $G^{1523} \rightarrow A$ or a $U^{1524} \rightarrow C$ mutation (Fig. 4, mutants III and IV, respectively). Binding efficiencies relative to that shown by the wild-type sequence were determined by trichloroacetic acid precipitation of the material removed from the reaction mixture with avidin-agarose beads after incubation of ^{35}S-labeled L25 with wild type and each of the mutant fragments. Data for mutants I and II, obtained in the same way, are also shown (Table 1).

The $G^{1523} \rightarrow A$ transition (mutant III) reduced the efficiency of L25 binding to a barely detectable level. The $U^{1524} \rightarrow C$ transition had a less severe, but still considerable negative effect, decreasing binding efficiency to ≈30% of the wild-type value. As shown by the results obtained with mutant II (Fig. 3 and Table 1), the presence of an adenosine and a cytidine at positions 1523 and 1524, respectively, did not significantly affect the ability of the rRNA fragment to associate with the L25 protein, provided complementarity with the residues at positions 1611 and 1612 was maintained. Consequently, the negative effect on protein binding of the individual changes present in mutants III and IV must be due to disruption of base pairing, rather than the sequence changes *per se*. Thus, the binding data for mutants III and IV constitute clear experimental support for the existence and functional importance of both base pairs of the tertiary interaction in domain III of the large subunit rRNA.

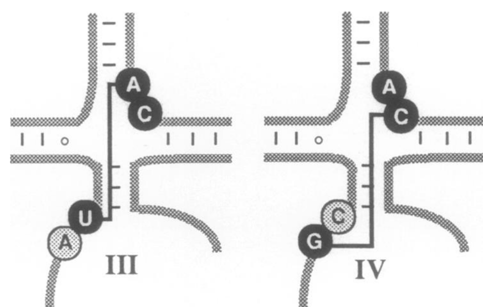

FIG. 4. Schematic representation of the structure of the single disruption mutants. Mutated residues are shaded. Possible pairing is indicated by lines connecting the residues involved in the tertiary interaction.

The destabilization of the tertiary interaction caused by disruption of either the pair between positions 1523 and 1612 or that between positions 1524 and 1611 is likely to cause a shift in the equilibrium between the biologically active and the unfolded states of the rRNA fragment in favor of the latter. However, because the binding assay is carried out with a large excess of RNA over protein, we deem it unlikely, at least for mutant IV, that the reduction in protein binding stems directly from this shift. A more plausible explanation is that the mutant fragment folds into a conformation having a lower affinity for the ribosomal protein because of imperfect alignment of multiple structural features required for docking of the protein. This would be in agreement with the suggestion of Egebjerg *et al.* (24) that the tertiary interaction serves to juxtapose the terminal loop of helix 49 and the single-stranded region connecting helices 46 and 47, where chemical probing of an EL23-23S rRNA complex (24) has shown possible contact points for the ribosomal protein to be clustered (cf. Fig. 2). Experimental proof for this hypothesis, however, will require determination of the binding constants for the mutant and wild-type rRNA fragments, which, due to the limited amounts of synthetic L25 that can be prepared, is not possible with the present *in vitro* binding assay.

In conclusion, our data strongly support the existence of a doublet tertiary interaction in domain III of the large subunit rRNA that has previously been proposed on the basis of phylogenetic covariance analysis. This interaction is of vital functional importance since it ensures the correct conformation of the binding site for a ribosomal protein whose failure to assemble has been shown by this laboratory (25–27) to be lethal for the yeast cell.

Table 1. Relative *in vitro* binding efficiency of ribosomal protein L25 to the various mutant forms of its binding region in 26S rRNA

Fragment	Mutation(s)	Relative binding efficiency, %
Wild type	—	100
Mutant I	$G^{1523}U^{1524} \rightarrow AC$	1 (±1)
Mutant II	$G^{1523}U^{1524} \rightarrow AC$, $A^{1611}C^{1612} \rightarrow GU$	102 (±8)
Mutant III	$G^{1523} \rightarrow A$	4 (±3)
Mutant IV	$U^{1524} \rightarrow C$	28 (±4)

Efficiency of binding of synthetic L25 to each of the mutant 26S rRNA fragments was determined by incubating synthetic [^{35}S]methionine-labeled L25 with excess biotinylated wild-type or mutant 26S rRNA fragment. Protein–RNA complex was removed from the reaction mixture with avidin-agarose beads and the amount of protein bound to the RNA was determined by trichloroacetic acid precipitation of the material attached to the beads (21). Binding is expressed as percentage of the binding to wild-type fragment, which was set at 100%. Each value is the average of four assays. The standard deviation is given in parentheses.

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