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Replacement of the L11 binding region within *E.coli* 23S ribosomal RNA with its homologue from yeast: *in vivo* and *in vitro* analysis of hybrid ribosomes altered in the GTPase centre

Jill Thompson, Wouter Musters[1], Eric Cundliffe[2] and Albert E.Dahlberg[3]

Department of Biochemistry, University of Leicester, Leicester LE1 7RH, UK, [1]Unilever Research Laboratory, 3130 AC Vlaardingen, The Netherlands and [3]Section of Biochemistry, Brown University, Providence, RI 02912, USA

[2]Corresponding author

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Replacement of the protein L11 binding domain within *Escherichia coli* 23S ribosomal RNA (rRNA) by the equivalent region from yeast 26S rRNA appeared to have no effect on the growth rate of *E.coli* cells harbouring a plasmid carrying the mutated *rrnB* operon. The hybrid rRNA was correctly processed and assembled into ribosomes, which accumulated normally in polyribosomes. Of the total ribosomal population, <25% contained wild-type, chromosomally encoded rRNA; the remainder were mutant. The hybrid ribosomes supported GTP hydrolysis dependent upon *E.coli* elongation factor G, although at a somewhat reduced rate compared with wild-type particles, and were sensitive to the antibiotic, thiostrepton, a potent inhibitor of ribosomal GTPase activity that binds to 23S rRNA within the L11 binding domain. That thiostrepton could indeed bind to the mutant ribosomes, although at a reduced level relative to that seen with wild-type ribosomes, was confirmed in a non-equilibrium assay. The rationale for the ability of the hybrid ribosomes to bind the antibiotic, given that yeast ribosomes do not, was provided when yeast rRNA was shown by equilibrium dialysis to bind thiostrepton only 10-fold less tightly than did *E.coli* rRNA. The extreme conservation of secondary, but not primary, structure in this region between *E.coli* and yeast rRNAs allows the hybrid ribosomes to function competently in protein synthesis and also preserves the interaction with thiostrepton.

Key words: EF G/protein L11 binding site/ribosomal RNA/thiostrepton/yeast–*E.coli* hybrid ribosomes

Introduction

The binding site for protein L11 within 23S ribosomal RNA (rRNA) of *Escherichia coli* (residues 1052–1112; Schmidt *et al.*, 1981) has been implicated in a number of functions associated with protein synthesis. Transfer RNA bound in the A site of the larger ribosomal subunit protects residues 1068 and 1071, albeit weakly, from chemical attack (Moazed and Noller, 1989) and a direct interaction between this region of the RNA and elongation factor G (EF G) has been revealed by cross-linking studies (Sköld, 1983) as well as by chemical footprinting (Moazed *et al.*, 1988). The antibiotic, thiostrepton, an aggressive inhibitor of ribosome-based GTP hydrolysis associated with elongation factors (for review see Gale *et al.*, 1981), binds to a synthetic 61mer oligonucleotide, that has the sequence of the L11 binding site, with almost the same avidity ($K_{diss} \sim 10^{-6}$ M) as to total 23S rRNA (Thompson and Cundliffe, 1991). Within that oligonucleotide, residue A1067 plays a pivotal role in the binding site for the drug; specific methylation of this nucleoside abolishes thiostrepton binding (Thompson *et al.*, 1982) and site-directed mutagenesis that affects this residue attenuates the interaction (Thompson *et al.*, 1988).

The functional importance of the L11 binding domain of 23S rRNA is underscored by the extreme conservation of its secondary structure across all three primary kingdoms, in chloroplast and mitochondrial rRNAs, and even in the minimal rRNAs from trypanosomal mitochondria. Indeed, the secondary structure is conserved to such an extent that *E.coli* ribosomal protein L11 binds to 23S-like rRNA from archaebacteria (Beauclerk *et al.*, 1985) and from yeast and mouse (El-Baradi *et al.*, 1987). Conversely, the yeast homologue of protein L11 (ribosomal protein L15) also binds to the equivalent region of mouse rRNA (El-Baradi *et al.*, 1987). Recently, the functional conservation of this rRNA domain has been examined directly by exchanging the yeast sequence for the *E.coli* equivalent in a tagged rRNA transcriptional unit. The mutant rRNA accumulated normally in yeast ribosomes and Northern blot analysis demonstrated that the polysome profile was indistinguishable from that of the wildtype (Musters *et al.*, 1991), indicating that ribosomal function was not grossly compromised. Further studies in that system were precluded due to the very high proportion of wild-type ribosomes present in the mutant strain. The converse experiment reported here, in which the yeast RNA sequence, corresponding to that of the L11 binding site, was introduced into *E.coli* rRNA, allowed hybrid ribosomes to be studied both *in vivo* and *in vitro*.

Results

Construction of plasmid pVMI

The exchange of DNA within the *rrnB* operon, encoding helices 39 and 40 of *E.coli* 23S rRNA (numbering according to the model of Brimacombe *et al.*, 1988), for the homologous region of the *Saccharomyces cerevisiae* 26S rRNA gene, was accomplished in several steps. First, the 679 bp *Sac*I–*Hae*III fragment of the 23S rRNA gene of the *rrnB* operon contained in pKK3535 (Brosius *et al.*, 1981) was fused with the 114 bp *Hae*III–*Sph*I fragment taken from pDVE (Vester and Garrett, 1987) and introduced between the *Sac*I and *Sph*I sites of pUC19 (Figure 1). The fusion of the *Hae*III-generated ends of these fragments created a *Stu*I site into which was inserted a 74 bp synthetic DNA fragment that restored helix 38 of *E.coli* 23S rRNA and introduced the sequence encoding helices 39 and 40 of *S.cerevisiae* 26S rRNA. The resulting 867 bp *Sac*I–*Sph*I fragment was used to replace the analogous fragment of plasmid pDVE, resulting in plasmid pWM1 (Figure 1).

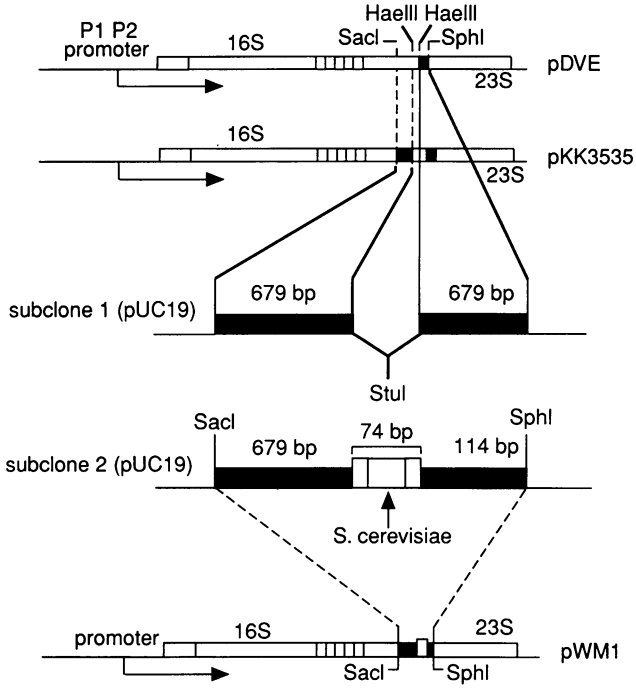

Fig. 1. Construction of plasmid pWM1.

Processing and assembly of mutant rRNA into active particles

Introduction of plasmid pWM1 (Figure 1) into *E.coli* DH1 cells appeared to have no effect on the growth rate in LB broth (data not shown). To exclude the trivial possibility that the hybrid rRNA gene was not being transcribed, processing of the mutant rRNA and its assembly into ribosomal particles was assessed in a maxicell system (Stark *et al.*, 1982). Analysis of ^{32}P-labelled, plasmid-encoded rRNA, in composite agarose–acrylamide gels, revealed that the mutant RNA was incorporated into ribosomal particles and polysomes in an apparently normal manner (data not shown). This result was confirmed when 23S rRNA was extracted from ribosomes and polyribosomes (separated by sucrose density gradient centrifugation) and sequenced using reverse transcriptase. The yeast sequence was revealed to such an extent that the wild-type sequence, contributed by chromosomally encoded rRNA, was barely distinguishable (data not shown). In order to quantitate the proportion of mutant to wild-type ribosomes, the rRNA was analysed using a modification of the primer extension method (Sigmund *et al.*, 1988). A deoxyoligonucleotide complementary in reverse to the 23S rRNA sequence (1100–1119), was labelled at its 5′ end and extended by reverse transcriptase using RNA from the mutant strain as a template. In a series of primer extension reactions, dGTP or dCTP was replaced in turn by its corresponding ddNTP in the incubation mix. In the example outlined in Figure 3, where ddGTP was included in place of dGTP, the primer extending into the *E.coli* sequence incorporated eight residues (including the ddGTP), whereas only three residues were incorporated when extending into the yeast sequence (see Figure 2). The relative amounts of the two species of extended primer reflects the relative abundance of 23S rRNA that has the *E.coli* and yeast sequences within the region of interest. The extended products were separated in a 12% polyacrylamide–8 M urea gel, subjected to autoradiography and the film analysed

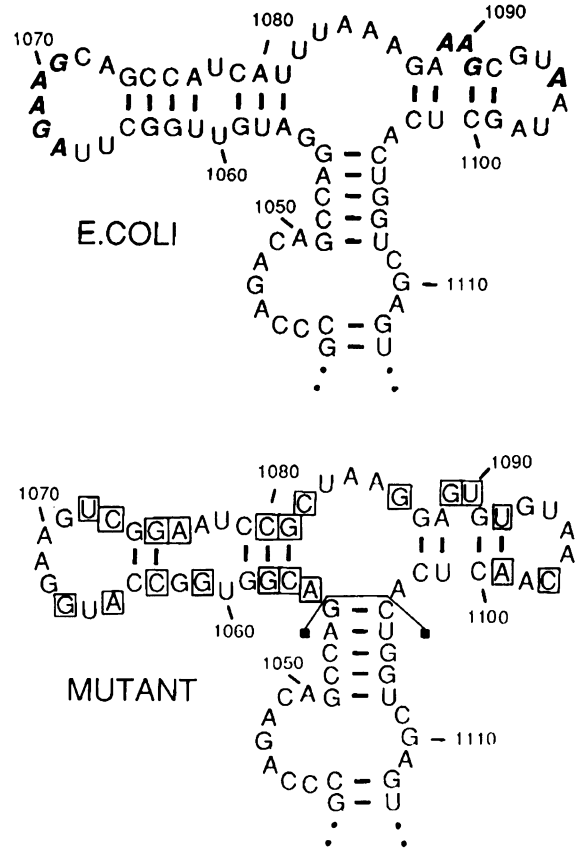

Fig. 2. Sequence and secondary structure of the L11 binding domain of 23S rRNA. Wild-type RNA encoded by the *rrnB* operon of *E.coli* is compared with that from the *E.coli*–yeast hybrid construct that has the homologous sequence from *S.cerevisiae* between residues 1056 and 1103. Nucleosides which differ between the yeast and *E.coli* sequences are enclosed in squares in the hybrid (i.e. mutant) sequence. Residues most strongly protected by thiostrepton from chemical attack are shown in bold italics in the *E.coli* sequence.

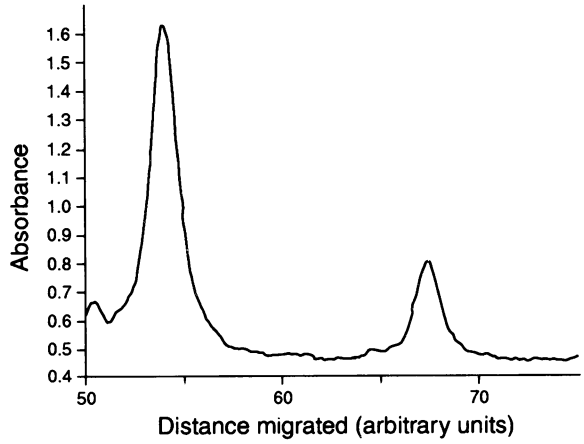

Fig. 3. Primer extension analysis of the rRNA content of ribosomes from *E.coli* carrying pWM1. Reverse transcriptase RNA sequencing was carried out using the deoxyoligonucleotide 5′ GAGTGACCAGCTCAGCCGGA 3′, which is complementary to the rRNA sequence immediately downstream of the hybrid junction site. In a series of reactions, each dNTP was replaced in turn by its corresponding ddNTP and priming was with 5′ ^{32}P-labelled oligonucleotide. Shown here is a densitometric scan of an autoradiogram of the reaction products when dGTP was replaced by ddGTP. Fractionation was in a 12% acrylamide–8 M urea gel. The slower migrating, larger peak represents the hybrid cDNA product.

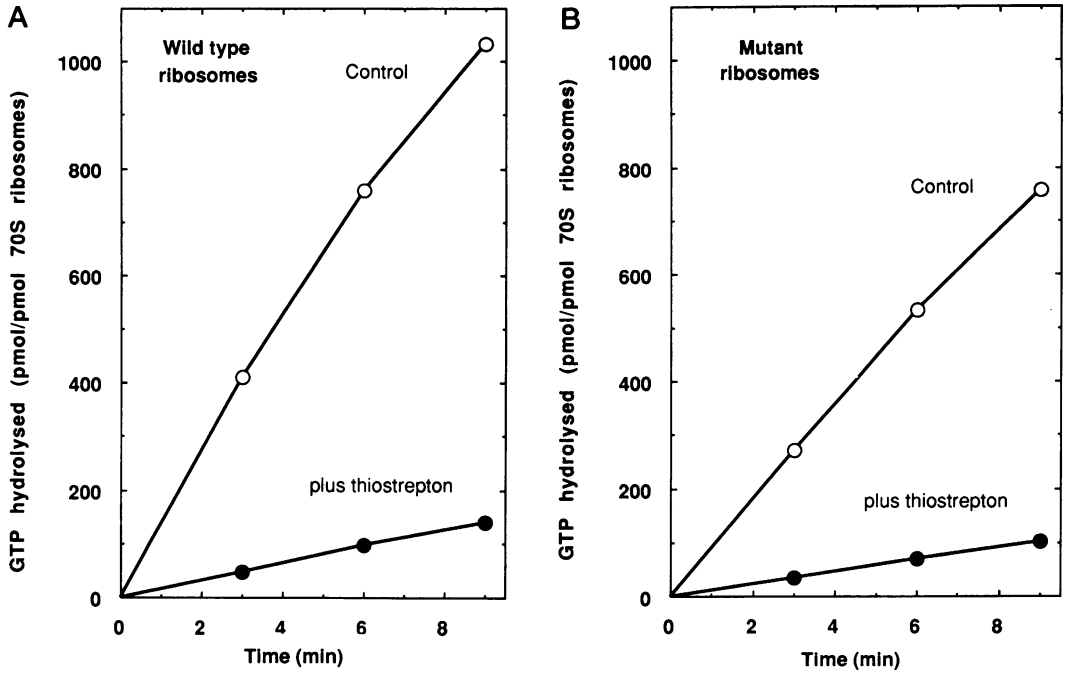

Fig. 4. Uncoupled hydrolysis of GTP by ribosomes and *E.coli* EF G. *E.coli* 70S ribosomes, from cells carrying (**A**) pKK3535 or (**B**) pWM1 were preincubated with DMSO (○) or with a 3-fold molar excess of thiostrepton (●) prior to addition of [γ-^{32}P]GTP and EF G. Data summarize the results of three experiments with each preparation of ribosomes.

densitometrically. A typical scan (Figure 3) indicated that ~77% of the ribosomes contained hybrid 23S rRNA and this value did not vary significantly (77 ± 4%) in 10 estimates involving three different preparations of ribosomes. We were therefore reassured that pWM1 was stable in this strain and that the proportion of mutant 23S RNA was unlikely to vary markedly from day to day. The steady-state level of rRNA derived from pWM1 is high, but not unusual, as it is expressed from the strong *rrnB* promoters P1 and P2 (Vester and Garrett, 1987).

In vitro EF G-dependent GTP hydrolysis

The ability of the mutant ribosomes to support a normal growth rate raised an interesting question concerning the presumed ability of the hybrid ribosomes to interact with prokaryotic EF G. Accordingly, mutant and wild-type 70S particles were examined for their ability to support GTP hydrolysis in the presence of purified *E.coli* EF G but in the absence of other factors normally required for protein synthesis. The rates of GTP hydrolysis were essentially linear over the first 9 min of the reaction (Figure 4) and, given that the proportion of mutant 70S ribosomes had already been determined, it could be calculated that the mutant ribosomes were performing at ~66% of the wild-type rate. In these experiments, the amount of ribosomes in the reaction mixture was deliberately limiting. It is not known what reduced rate of GTP hydrolysis *in vitro* would result in rate limitation for protein synthesis *in vivo*, but a reduction of one-third, such as that observed here, clearly did not do so. Somewhat surprising was the effect of the antibiotic, thiostrepton, on the reaction. This drug is a potent inhibitor of protein synthesis in prokaryotes, but the ribosomes of yeast are totally resistant to its action (J.Thompson, unpublished data). Given that the binding site for thiostrepton lies within the prokaryotic 23S rRNA fragment exchanged in pWM1 for that from yeast 26S rRNA, the ability of the antibiotic to inhibit EF G-dependent GTP hydrolysis by the mutant ribosomes was unexpected; indeed, the level of inhibition was very similar to that seen with wild type ribosomes. Given the rate of GTP hydrolysis in the absence of the antibiotic and the fact that <25% of the ribosomes were wild-type, the interpretation that the mutant 70S ribosomes were interacting with thiostrepton was inescapable.

Thiostrepton binding

To investigate in more detail the interaction of thiostrepton with the mutant ribosomes, the binding of labelled antibiotic to 70S particles was examined in a non-equilibrium assay. The results (Figure 5) clearly indicated that the mutant 70S ribosomes bound the drug at ~40% of the level of wild-type particles (taking into account the relative proportion of wild-type and mutant ribosomes). Even by the much more sensitive assay of equilibrium dialysis, 80S particles from yeast did not bind thiostrepton detectably (data not shown). This apparent paradox was resolved when the binding of [^{35}S]thiostrepton to total rRNA from either *E.coli* wild-type or yeast was examined by equilibrium dialysis (Figure 6). Yeast rRNA binds the drug with an affinity only one order of magnitude lower than that observed with *E.coli* rRNA. Presumably in the yeast ribosomes, protein L15 (the homologue of *E.coli* protein L11) and/or other proteins occlude the potential binding site for thiostrepton.

Discussion

The exchange within *E.coli* 23S rRNA of the L11 binding region for the homologous sequence from yeast, equivalent to the introduction of a cluster of 20 point mutations, elicits a resounding lack of effect, at least *in vivo*. Given the high proportion of mutant ribosomes within the manipulated strain, this result amply demonstrates that changing the primary sequence in this way (without radically altering the

secondary structure) did not prevent the adequate functioning of the mutant ribosomes or their recognition by non-ribosomal factors. Equally, although not directly examined, it is highly unlikely that the incorporation of protein L11 into the hybrid ribosomes was affected. Mutants of *E.coli* or *Bacillus megaterium*, devoid of protein L11 or its equivalent, are grossly impaired in growth rate and their ribosomes are severely defective in EF G-dependent GTP hydrolysis *in vitro* (Cundliffe *et al.*, 1979; Stark and Cundliffe, 1979; Stöffler *et al.*, 1980). And, in any case, the demonstrated ability of *E.coli* protein L11 to bind to yeast 26S rRNA (El-Baradi *et al.*, 1987) implies that attachment of the protein would presumably not be affected in the hybrid ribosomes. It was only when the mutant ribosomes were examined *in vitro* that some consequences of the RNA sequence exchange were revealed. The overall rate of GTP hydrolysis, mediated by EF G, was somewhat lower than that with wild-type ribosomes.

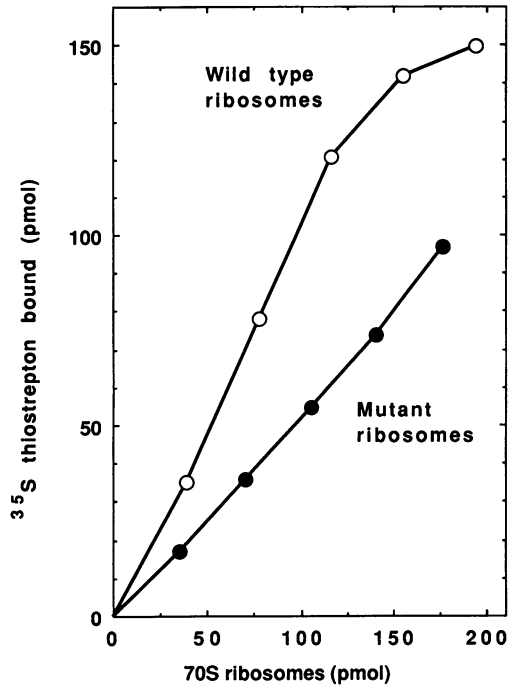

Fig. 5. Binding of thiostrepton to ribosomes. Ribosomes from *E.coli* carrying either pKK3535 (○) or pWM1 (●) were incubated with [^{35}S]thiostrepton (75 pmol; 400 c.p.m./pmol). Unbound antibiotic was removed by adsorption to activated charcoal, which was precipitated using a microcentrifuge.

Nucleotides within 23S rRNA, protected by EF G from chemical attack, are grouped in two clusters (Moazed *et al.*, 1988). The majority are in the universally conserved loop (residues 2653–2667), which contains the site of action of the cytotoxin α-sarcin (Endo and Wool, 1982), whereas the remainder (residues A1067 and A1069) are in the L11 binding domain studied here. It has been suggested that tertiary interaction between these two RNA domains may create the so-called GTPase centre of the ribosome (for review see Cundliffe, 1990) and, on that basis, changes introduced into one of these regions might well have been expected to hold functional consequences in the absence of compensating changes in the other. It is therefore of particular interest that the changes generated here within the L11 binding domain, by substitution of the yeast sequence, did not seriously compromise ribosomal function.

The response of the hybrid ribosomes to thiostrepton was unexpected, given that yeast ribosomes are totally resistant to the action of the drug when examined in cell-free protein-synthesizing systems. However, the present observation that yeast 26S rRNA possesses a binding site for thiostrepton, which is presumably masked by protein(s) in the fully

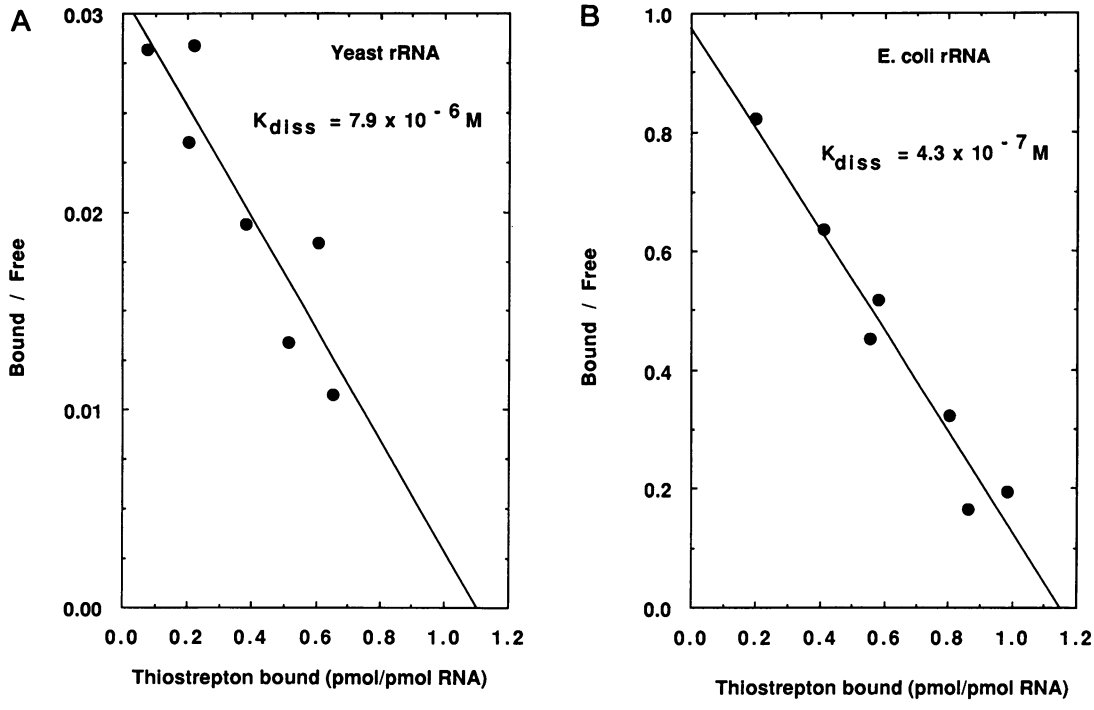

Fig. 6. Binding of thiostrepton to total rRNA. Equilibrium dialysis was used to quantitate the binding of [^{35}S]thiostrepton to 100 pmol of rRNA extracted from ribosomes of (**A**) *E.coli* and (**B**) *S.cerevisiae*. Inputs of labelled antibiotic ranged from 25 to 200 pmol with *E.coli* RNA and 100–1000 pmol with yeast RNA. Data represent the means of three experiments.

assembled particle, reconciles the apparent paradox. More importantly, it raises interesting questions concerning the nature of the thiostrepton binding site within 23S (and now, 23S-like) rRNA. Residue A1067, methylation of which abolishes the binding of thiostrepton to isolated 23S RNA or to ribosomes, is evidently not the sole point of attachment of the drug. This was demonstrated directly when residue 1067 was altered by site-directed mutagenesis (Thompson *et al.*, 1988). Thus, the A to G mutation caused only a 10-fold reduction in the affinity of thiostrepton for 23S RNA and, even when pyrimidine nucleosides were introduced at that site, thiostrepton binding was still detectable (Thompson and Cundliffe, 1991). It may not, however, be a passing coincidence that the dissociation constant ($\sim 10^{-6}$ M) for the interaction of thiostrepton with *E.coli* 'G1067' RNA was very similar to that reported here with yeast 26S rRNA, which also has a G at the equivalent position. Chemical footprinting experiments (Egebjerg *et al.*, 1989), whilst highlighting A1067 as one of the residues principally protected by thiostrepton, also revealed protection of at least eight other residues clustered at both ends of the L11 binding domain of *E.coli* 23S RNA. Of those nine residues most strongly protected by the drug (indicated in Figure 2), all but three are retained unchanged in the hybrid *E.coli*–yeast sequence, strongly suggesting that they form the core of the thiostrepton binding site.

In an alternative approach to studying the structure of *E.coli* 23S RNA necessary for interaction with thiostrepton, a set of mutations was generated that, collectively, altered the RNA sequence at every position between residues 1051 and 1108 (Ryan *et al.*, 1991). This was done using a 23S RNA gene fragment corresponding to residues 1029–1122. On the one hand, many such changes caused a dramatic weakening of thiostrepton binding, even when the secondary structure was ostensibly preserved. Conversely, 20 out of 58 changes had relatively little effect on drug binding. Here, without loss of function or thiostrepton sensitivity, we have introduced 20 changes simultaneously into full length 23S rRNA, which precludes detailed comparisons between our data and theirs. However, our demonstration that thiostrepton binds to yeast 26S rRNA is at variance with their supposition that insensitivity of yeast ribosomes to the drug derives from the RNA sequence.

An underlying belief in the importance of determining the structure of rRNA from diverse sources has been that conserved regions should be functionally important. The corollary of this is that conserved regions of rRNA should be interchangeable, without too drastic an effect upon ribosomal function, as shown here. In pursuing our argument, we have assumed, on the basis of considerable experimental evidence, that the L11 binding domain of 23S RNA is involved in GTP hydrolysis associated with EF G. However, our case would still rest if some other function were involved, additionally or alternatively. It is, in essence, a general argument, also applicable to other conserved domains within ribosomal RNA.

Materials and methods

Preparation of ribosomes and ribosomal RNA

Ribosomes were prepared from log-phase cells of *E.coli* DH1, containing either pWM1 (carrying the hybrid *rrnB* operon) or pKK3535 (wild-type *rrnB* operon), and from *S.cerevisiae* D273-10B, as previously described (Cundliffe *et al.*, 1979). Total rRNA was obtained by repeated extraction of ribosomes with phenol–chloroform followed by ethanol precipitation. The RNA was dried under vacuum, dissolved in HRS buffer (10 mM HEPES–KOH, pH 7.6, 10 mM $MgCl_2$, 50 mM NH_4Cl, 6 mM 2-mercaptoethanol) and stored at −70°C. Plasmid-encoded, ^{32}P-labelled ribosomes were prepared using the maxicell system and analysed by composite gel electrophoresis, as described previously (Stark *et al.*, 1982).

Sequencing of rRNA and primer extension analysis

Reverse transcriptase sequencing across the L11 binding region of mutant and wild-type rRNAs was primed using an oligonucleotide (20mer) complementary in reverse to residues 1100–1119 of *E.coli* 23S rRNA. Sequencing reactions were carried out according to standard protocols (Sambrook *et al.*, 1989). The ratio of hybrid to wild-type RNA in each ribosome preparation from *E.coli* (pWM1) cells was determined by a modification of the primer extension method (Sigmund *et al.*, 1988). Reverse transcriptase sequencing reactions were carried out as above but with each dNTP replaced in turn with the appropriate ddNTP. The 5′-dephosphorylated primer was labelled using [γ-^{32}P]ATP (>10 Ci/mmol, Amersham International) and T4 polynucleotide kinase. Extension products were separated in 12% acrylamide–8 M urea ultra-thin gels, subjected to autoradiography and quantitated using an LKB Ultroscan XL densitometer.

Uncoupled hydrolysis of GTP by ribosomes and EF G

Uncoupled GTP hydrolysis was assayed as previously described (Stark and Cundliffe, 1979) except that ribosomes (2 pmol/assay) were from *E.coli* strains carrying either pWM1 or pKK3535. Where appropriate, ribosomes were preincubated with thiostrepton or dimethylsulphoxide (DMSO) (in controls) for 10 min at room temperature prior to the addition of excess EF G and [γ-^{32}P]GTP (9 mmol/assay).

Preparation and binding of [^{35}S]thiostrepton

Radiolabelled thiostrepton was prepared as described (Dixon *et al.*, 1975) except that *Streptomyces laurentii* was used in place of *Streptomyces azureus* as the producing organism. Binding of the drug to *E.coli* ribosomes was determined using a non-equilibrium assay (Thompson *et al.*, 1979). Equilibrium dialysis was employed to quantitate binding to rRNA species and to yeast 80S ribosomes (Thompson and Cundliffe, 1991).

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