Species Barrier to RNA Recognition Overcome with Nonspecific RNA Binding Domains*

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We show here that nonspecific RNA-protein interactions can significantly enhance the biological activity of an essential RNA-protein complex. Bacterial glutaminyl-tRNA synthetase poorly aminoacylates yeast tRNA and, as a consequence, cannot rescue a knockout allele of the gene for the yeast homologue. In contrast to the bacterial protein, the yeast enzyme has an extra appended domain at the N terminus. Previously, we showed that fusion of this yeast-specific domain to the bacterial protein enabled it to function as a yeast enzyme in vivo and in vitro. We suggested that the novel yeast-specific domain contributed to RNA interactions in a way that compensated for the poor fit between the yeast tRNA and bacterial enzyme. Here we establish that the novel appended domain by itself binds nonspecifically to different RNA structures. In addition, we show that fusion of an unrelated yeast protein, Arc1p, to the bacterial enzyme also converts it into a functional yeast enzyme in vivo and in vitro. A small C-terminal segment of Arc1p is necessary and sufficient for this conversion. This segment was shown by others to have nonspecific tRNA binding properties. Thus, nonspecific RNA binding interactions in general can compensate for barriers to formation of a specific and essential RNA-protein complex.

Interactions between proteins and nucleic acids contain both specific and nonspecific components. The former include interactions between amino acid side chains and functional groups on bases, whereas nonspecific contacts with nucleic acids are generally directed toward the sugar and phosphate backbone (1–3). From the standpoint of functional relevance, the relative importance of nonspecific versus specific interactions has been difficult to evaluate. Most work has demonstrated the importance of particular specific interactions, generally by experiments with mutants where well defined contacts are replaced or manipulated (4, 5). However, less is known about the role of nonspecific contacts and their contribution toward the formation and function of specific complexes. With this in mind, we asked whether, in general, nonspecific protein-RNA interactions could restore function in vivo and in vitro to a weak complex that required high specificity to be functional.

We show here that nonspecific RNA binding domains can significantly influence the functional activity of a highly specific enzyme-RNA interaction. This influence is simply achieved by fusing a nonspecific RNA binding domain to a heterologous target protein whose function requires a specific RNA interaction. The heterologous target protein is itself inactive in the organism chosen. The idea was to rescue its activity by fusion of a new RNA binding element.

For this work, we used an aminoacyl-tRNA synthetase system that could be investigated both in vivo and in vitro (6). Because the synthetases are essential proteins, the investigation of specific interactions in vivo provided us with a stringent test for functional enzyme-RNA complexes. For example, complexes that were either either nonfunctional or lacked high specificity (i.e., resulted in misacylation of tRNAs) would fail to support normal cell growth (7–9).

These investigations are built upon recent results with the yeast and Escherichia coli glutaminyl-tRNA synthetase (GlnRS) system (Fig. 1). The entire 551 amino acids of the monomeric E. coli glutaminyl-tRNA synthetase can be aligned with the 809-amino acid polypeptide of the Saccharomyces cerevisiae glutamine enzyme (10). The sequences of the aligned portions are highly conserved, having an identity of about 40%. The extra length of the yeast enzyme is mostly at the N terminus in the form of an appended domain (Ad) of 228 amino acids. This domain is missing from prokaryote glutaminyl-tRNA synthetases. Like the appended domain found in other yeast tRNA synthetases, the one fused to the glutamine enzyme is lysine-rich and therefore highly basic. Consequently, this domain is expected to have anion-binding properties (11, 12).

Because of critical nucleotide differences between yeast and E. coli glutamine tRNAs, these closely homologous enzymes do not cross-acylate their respective tRNAs, that is, aminoacylation is species-specific (6). As a consequence, the expression of the E. coli enzyme in S. cerevisiae does not rescue a knockout allele of the gene for yeast GlnRS. But fusion of the appended domain (from the yeast protein) to the body of the bacterial synthetase gave a chimeric protein that could aminoacylate yeast tRNA in vitro. Moreover, expression of the fusion protein in yeast rescued the growth defect of the strain harboring the knockout allele of the gene for the yeast enzyme (6). Because the fusion protein had a reduced $K_a$ for yeast tRNA transcript relative to the nonfused E. coli enzyme, we speculated that the appended domain had inherent RNA binding activity, probably because of its highly basic composition. Thus, when fused to the E. coli protein, the added RNA binding affinity overcame the barrier to species-specific aminoacylation.

In this work we cloned the 228-amino acid appended domain of yeast GlnRS and directly tested and established its RNA binding properties. We also investigated whether the properties of this domain were unique or whether an unrelated and known nonspecific tRNA binding protein could confer function in yeast on E. coli glutaminyl-tRNA synthetase. To address this question, we fused an unrelated RNA binding domain to the E.

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The abbreviations used are: GlnRS, glutaminyl-tRNA synthetase; Ad, appended domain; 5-FOA, 5-fluoroorotic acid.
coli enzyme, as we had done with the appended domain of yeast glutaminyl-tRNA synthetase. The rationale was that the ability of nonspecific RNA binding domains to affect specific interactions may be general and that the general nature of these effects might be demonstrated with an RNA binder unrelated to those associated with the glutamine system.

**MATERIALS AND METHODS**

**Aminoacylation Assays**—Aminoacylation reactions were carried out at ambient temperature in a buffer containing 30 mM HEPES (pH 7.5), 25 mM KCl, 15 mM MgCl₂, 5 mM dithiothreitol, 4 mM ATP, and 30 μM glutamine (5 μM [3H]glutamine; Amersham Pharmacia Biotech) (6). Reactions were quenched by spotting 10-μl aliquots on Whatman filters soaked in 5% trichloroacetic acid and 1 mM glutamine. The filters were washed three times, 15 min each, in ice-cold 5% trichloroacetic acid before liquid scintillation counting. The preparation of yeast tRNA^{Gln} (CUG) transcript was as described previously (6).

**Complementation**—A strain (EFW6) with a partial deletion of gln4 was constructed as described previously (6). Briefly, an 835-base pair EcoRI fragment (encoding residues 384–662) of gln4 was deleted and replaced by TRP1 gene, using standard genetic methods with a ura3 yeast strain. The growth of EFW6 gln4Δ:TRP1 strain is maintained by plasmid pEFW111 which contains GLN4 and the selectable marker URA3. The complementation assay was performed by introducing into EFW6 a second plasmid containing a test gene, such as an Arc1p-EcGlnRS fusion in a yeast shuttle vector, the full-length Arc1p-EcGlnRS fusion in a yeast shuttle vector, the full-length Arc1p- EcGlnRS fusion in a yeast shuttle vector, the full-length Arc1p- EcGlnRS fusion in a yeast shuttle vector, the full-length Arc1p- EcGlnRS fusion in a yeast shuttle vector, the full-length Arc1p- EcGlnRS fusion in a yeast shuttle vector.

**Construction and Purification of Chimeric Proteins**—For constructing an Arc1p-EcGlnRS fusion in a yeast shuttle vector, the full-length open reading frame of arc1 (the gene encoding Arc1p; Ref. 13) was amplified from yeast genomic DNA by polymerase chain reaction. An NdeI-NdeI fragment from arc1 was cloned in-frame into the NdeI site at the start of the reading frame for E. coli glnS. (The coding sequence for the C terminus of E. coli glnS was tagged previously (6) with a sequence encoding the 12CA5 epitope.) The resulting DNA containing arc1-glnS was subsequently used as a template for polymerase chain reaction amplification of individual Arc1p domains (NΔArc1p, MΔArc1p, CΔArc1p, and (M+C)ΔArc1p). Fusion of the DNA encoding these individual domains to the coding region of E. coli glnS followed a similar approach to that for creating the arc1-glnS fusion. Expression in yeast of E. coli glutaminyl-tRNA synthetase as well as the fusion proteins was monitored by immunoblotting with the anti-12CA5 antibody (14) (Roche Molecular Biochemicals).

For purification of E. coli glutaminyl-tRNA synthetase and the fusion proteins, constructions similar to those described above were made in the E. coli expression vector pQE70 (Qiagen, Chatsworth, CA). Briefly, a new NdeI site was introduced into the previously described clone (6) to replace the Sp6I site at the start of the coding sequence for glnS that has a C-terminal sequence encoding the 12CA5 epitope and a His₆ tag. For this purpose, the native NdeI site in the vector was removed by mutagenesis (15). Subsequently, the NdeI-NdeI fragments of arc1 and its domains were separately cloned into the NdeI site at the start of the coding sequence for glnS. Standard protocols were used for expression and purification of E. coli glutaminyl-tRNA synthetase and the fusion proteins on a Ni-nitrilotriacetic acid affinity column (6) (Qiagen).

**Affinity Co-electrophoresis**—Protein-RNA interactions were assayed using affinity co-electrophoresis as described previously (16, 17). Crude yeast tRNA was purchased from Roche Molecular Biochemicals, and oligoA₆₅ was chemically synthesized on a Gene Assembler Special (Amersham Pharmacia Biotech). Pseudoknot RNA, a 28-mer RNA, is derived from the sequence of the RNA-dependent RNA polymerase mRNA of beet western yellow virus (18) and was generously donated by the laboratory of Professor Alexander Rich (Massachusetts Institute of Technology). The preparation of yeast tRNA^{Gln} (CUG) transcript was as described previously (6). Various RNAs were labeled with 32P using polynucleotide kinase (New England Biolabs, Beverly, MA), after dephosphorylation with calf intestine phosphatase (New England Biolabs) (19). The purified appended domain (0.03–24 μM), E. coli GlnRS (0.01–72 μM), and fusion proteins (0.003–24 μM) were individually mixed with 5% polyacrylamide solution to prepare each mini-gel matrix. Radioactively labeled RNAs were loaded at an estimated concentration of 1 nM in 2-μl aliquots. Gels were run in a buffer containing 0.5× TBE (45 mM Tris borate and 1 mM EDTA) with 50 mM NaCl at 20 °C at 50 V for 1 h. After electrophoresis, gels were dried and analyzed with a PhosphorImager (Molecular Dynamics). Dissociation constants (Kₐ) of the proteins and various RNAs were determined as described previously (20).

**RESULTS**

The Appended Domain of Yeast Glutaminyl-tRNA Synthetase Is an RNA Binding Domain—The N-terminal 228 amino acid polypeptide extension of yeast glutaminyl-tRNA synthetase co-electrophoresis gels have a protein gradient that increases from left to right in each panel. The protein concentrations across the gel were varied by successive 2-fold (left panel) or 3-fold (middle and right panels) dilutions. Therefore, the protein concentration gradient is logarithmic. Left, the appended domain binds to crude yeast tRNA. Middle, the appended domain binds to both yeast tRNA and a viral RNA pseudoknot. The pseudoknot RNA (28-mer) moved faster than yeast tRNA because of the size difference. Right, the appended domain binds to oligoA₆₅. In all panels, the RNA substrates moved in the direction indicated by the arrow.
tase was cloned (with a His₆ tag) into an E. coli expression vector. The protein expressed in E. coli was purified to apparent homogeneity on a Ni-nitrilotriacetic acid affinity column. The recombinant protein was then tested for its RNA binding properties, using polyacrylamide affinity co-electrophoresis. With this procedure, a horizontal gradient of protein is established throughout the gel. The radioactively labeled RNA is then electrophoresed through the protein gradient. Because the RNA is always in the presence of protein, complex formation is detected as a retardation in the rate of vertical migration of the RNA. This retardation, in turn, takes the form of a titration curve that can be interpreted in terms of an apparent dissociation constant (16, 17).

Using unfractionated, labeled yeast tRNA, and a horizontal gradient of the appended domain of yeast GlnRS, we observed a shift in migration of the tRNA (Fig. 2, left). The midpoint of this shift occurred at about 0.6 μM. All of the tRNA shifted in concert, showing that the interaction of the appended domain with these tRNAs was not particularly tRNA-specific. Consistent with this observation, we also observed binding of the recombinant appended domain to a synthetic 28-nucleotide RNA pseudoknot based on that found in the mRNA of the gene for the RNA-dependent RNA polymerase of beet western yellow virus. To investigate further the nonspecific RNA binding by the appended domain, we mixed pseudoknot RNA with unfractionated yeast tRNA that served as an internal standard. Binding to both RNAs could be seen in the gel assay (Fig. 2, middle). The apparent Kₐ for the complex with the pseudoknot is estimated as 0.6 μM, about the same as that for the complex with unfractionated yeast tRNA. Thus the RNA binding properties of the appended domain are not specific to tRNA.

The two tested RNAs (tRNA and pseudoknot RNA) have considerable secondary and tertiary structure. With this in mind, we also tested a single-stranded RNA ligand, oligo(A₃₅). Binding to oligo(A₃₅) was also seen, with an apparent Kₐ of about 1.8 μM (Fig. 2, right). Hence, the appended domain of yeast glutaminyl-tRNA synthetase can bind to single- and double-stranded RNA ligands, with roughly comparable affinities.

Collectively, these observations offer an explanation for the enhancement of binding of E. coli glutaminyl-tRNA synthetase to yeast tRNA^Gln, when the appended domain was fused to the E. coli protein (6). At the same time, given the broad, nonspecific binding of the appended domain to tRNAs, the data in Fig. 2 raise the question of whether an arbitrary nonspecific RNA binding domain could confer function in yeast on an E. coli tRNA synthetase.

The tRNA Binding Domain of Arc1p Enables E. coli GlnRS to Complement the Yeast Knockout Strain—A well studied tRNA binding protein is Arc1p, which was found in a genetic screen for the proteins that interact with Los1p. The latter is involved in general tRNA export and is associated with the nuclear pore complex (13, 21). On the basis of secondary structure prediction, Arc1p can be roughly divided into three domains. These are designated as N-terminal (N^Arc1p) (residues 1–131), Middle (M^Arc1p) (residues 132–200), and C-terminal (C^Arc1p) (residues 201–376) domains (13). The (M+C)^Arc1p domain is a nonspecific tRNA binding domain (22). The C^Arc1p domain also exhibits significant sequence homology to several other proteins in the sequence data-base libraries, including the human endothelial-monocyte activating polypeptide II (EMAP II) (54% identity), the C terminus of E. coli MetRS (54% identity), and the N terminus of the β chain of E. coli PheRS (29% identity) (13).

We fused the coding sequence of the full-length Arc1p to that of the E. coli glutamine enzyme. We also made separate fusions with the coding sequences of the N^Arc1p, M^Arc1p, and C^Arc1p domains to E. coli GlnRS (Fig. 3A). The resulting chimeras were tested for their ability to rescue the growth of EFW6, the yeast strain harboring the gln4 knockout allele. While native E. coli GlnRS (EcGlnRS) cannot complement the yeast knockout strain, attachment of full-length Arc1p or of (M+C)^Arc1p to E. coli GlnRS enabled the E. coli enzyme to rescue the growth defect of the yeast strain (Fig. 3B). Moreover, a chimera with only the C^Arc1p domain of Arc1p fused to the E. coli enzyme (C^Arc1p-EcGlnRS) also rescued the null strain. In contrast, neither the N^Arc1p or M^Arc1p domain fusions were active. (Western blot analyses with antibodies directed against the 12CA5 epitope showed that these noncomplementing fusion proteins were stably expressed in yeast (data not shown).) Thus, complementation of the yeast null strain requires that...
the RNA binding CArc1p domain be included in the fusion with E. coli GlnRS.

The RNA binding property of the appended domain of yeast GlnRS is therefore not unique in its ability to confer function (in yeast) to the E. coli enzyme. We surmise that addition of RNA binding capacity per se may be sufficient to convert the bacterial protein to one that is functional in yeast.

The tRNA binding Domain of Arc1p Enhances the Ability of Bacterial GlnRS to Bind to and Aminoacylate Yeast tRNA<sub>Gln</sub>—The in vivo complementation results (Fig. 3B) suggest that the attached CArc1p domain enables the chimeric protein to bind yeast tRNA and in turn aminoacylate yeast tRNA. To investigate whether the grafted domain reduces the dissociation constant of the E. coli enzyme for yeast tRNA, we again used affinity co-electrophoresis to determine the tRNA binding affinity of some of the fusion proteins that complement the yeast null strain. We purified (from E. coli) recombinant forms of E. coli GlnRS, a fusion protein of E. coli GlnRS with the Ad of the yeast enzyme, and a fusion of the E. coli protein with the (M+C)Arc1p domain. These three proteins were then tested for binding to a transcript of yeast tRNA<sub>Gln</sub>.

Fusion of either Ad of yeast GlnRS or of the (M+C)Arc1p domain substantially enhanced the binding of the E. coli protein to the yeast tRNA<sub>Gln</sub> transcript (Fig. 4). For example, for the unfused E. coli enzyme, the apparent <i>K</i><sub>d</sub> was about 24 μM. When joined to either Ad of the yeast protein or to (M+C)Arc1p, the affinity increased dramatically to an apparent <i>K</i><sub>d</sub> ~ 0.06 μM (with the yeast Ad) and to <i>K</i><sub>d</sub> ~ 0.6 μM (with the (M+C)Arc1p).

We then investigated each of the fusion proteins in the aminoacylation assay with the yeast tRNA<sub>Gln</sub> transcript. As reported previously, E. coli GlnRS has weak activity toward the yeast tRNA<sub>Gln</sub> transcript (6). That activity is greatly enhanced by fusion of the appended domain of the yeast enzyme to E. coli GlnRS. We confirmed these results and then went on to show that fusion of (M+C)Arc1p to E. coli GlnRS also activates the E. coli enzyme for charging the yeast substrate (Fig. 5). We estimate that this enhancement brings the (M+C)Arc1p fusion with E. coli GlnRS to an activity on a yeast substrate within 2–5% of that of wild-type yeast GlnRS. This corresponds to an apparent free energy difference of ~2 kcal/mol. At the same time, the activity of the fusion enzyme is reduced ~2-fold toward E. coli tRNA, compared with wild-type E. coli GlnRS.) This enhancement of aminoacylation correlates with the complementation phenotypes shown in Fig. 3B.

DISCUSSION

The N-terminal appended domain of yeast GlnRS is the only polypeptide extension so far that has been shown to bind tRNA with a physiologically relevant affinity. It is not known whether other appended domains of yeast tRNA synthetases such as glutamyl-, isoleucyl-, lysyl-, methionyl-, and valyl-tRNA synthetases also exhibit similar RNA binding properties (23). However, the appended domains of yeast methionyl- and glutamyl-tRNA synthetases have been shown to interact with Arc1p, which itself binds tRNA and stimulates the aminoacylation activity of the associated methionine enzyme (13).

The observation that Arc1p and (M+C)Arc1p are equivalent when fused to E. coli GlnRS is consistent with the result of Simos et al. (22). Their experiments showed that Arc1p and (M+C)Arc1p had a similar nonspecific binding affinity to tRNA. However, in the work reported here, fusion of the C Arc1p domain was effective in activating E. coli GlnRS for aminoacylation in vivo, whereas the fusion with the M Arc1p domain was not effective (Fig. 3B). In the work of Simos et al. (22), the individual M Arc1p and C Arc1p domains each bound significantly less well to tRNA than did (M+C)Arc1p. Thus, our results suggest that the C Arc1p domain may be the more important contributor to nonspecific interactions with tRNA.

The apparent <i>K</i><sub>d</sub> for the complex of E. coli GlnRS with a transcript of yeast tRNA<sub>Gln</sub> is about 24 μM (Fig. 4). Approximately the same <i>K</i><sub>d</sub> was measured for the interaction of the E. coli protein with a mixture of yeast tRNAs (data not shown). This result suggests that E. coli GlnRS interacts nonspecifically with yeast tRNAs. And yet, fusion of either Ad from yeast GlnRS or (M+C)Arc1p from Arc1p gives a functional interaction of the catalytic body of the E. coli protein with yeast tRNA<sub>Gln</sub>, both in vivo (Fig. 3) and in vitro (Fig. 5). Even in the weak complex of E. coli GlnRS with tRNA<sub>Gln</sub> the substrate is probably bound to the active site in a productive complex. This
would account for the low rate of aminoacylation of the yeast tRNA_{Gln} complex by the unfused E. coli enzyme (Fig. 5). Fusion of either Ad from yeast GlnRS or of (M+C)Arc1p from Arc1p raises the affinity of the E. coli enzyme to the point that the rate of aminoacylation is then enhanced significantly. The dissociation constant of the complex is lowered about 400-fold by fusion of Ad from yeast GlnRS and about 40-fold by fusion of (M+C)Arc1p. These reductions in dissociation constants correspond to an additional apparent free energy of stabilization of the complex of ~3.6 and ~2.2 kcal mol^{-1}, respectively. By comparison, in a naturally occurring noncovalent complex between Arc1p and yeast MetRS, the apparent K_{on} for tRNA_{Met} is lowered more than 60-fold relative to MetRS alone.

If the effect of fusion of a nonspecific RNA binding domain is to lower the K_{on}, then the same effect might be achieved simply by raising the tRNA concentration in the aminoacylation assay with the unfused protein. In separate experiments, we found that raising the yeast tRNA_{Gln} concentration from 0.1 (the amount used in Fig. 5) to 2 μM reduced significantly the difference between the aminoacylation rates of the fused and unfused proteins (data not shown). This result further supports the idea that the body of E. coli GlnRS alone makes a weak but productive complex with yeast tRNA_{Gln} and that the effect of the nonspecific RNA binding domains is to bolster the stability of what is already an essentially active complex.

A stringent test for aminoacylation specificity is the in vivo complementation experiment (Fig. 3) because significant misacylations are known to be associated with toxic cell phenotypes (7–9). Thus, the robust growth of cells, whose only glutamyl-tRNA synthetase activity is derived from one of the E. coli fusion proteins, is consistent with few if any misacylations. Although E. coli GlnRS binds to unfractionated yeast tRNA, those complexes (other than the one with tRNA_{Gln}) are weak and most likely not productive. For example, we found little aminoacylation of unfractionated yeast tRNA when challenged with E. coli GlnRS (data not shown). This is consistent with the long standing observation that synthetase-tRNA complexes derive much of their specificity from k_{cat} rather than K_{on} discrimination (24, 25). Consequently, even when a noncognate complex is formed, aminoacylation is prevented by discrimination in the transition state of catalysis. Perhaps for this reason, expression of the unfused E. coli GlnRS in yeast is not toxic.

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