

# Promoting the Formation of an Active Synthetase/tRNA Complex by a Nonspecific tRNA-binding Domain\*

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Previous studies showed that valyl-tRNA synthetase of *Saccharomyces cerevisiae* contains an N-terminal polypeptide extension of 97 residues, which is absent from its bacterial relatives, but is conserved in its mammalian homologues. We showed herein that this appended domain and its human counterpart are both nonspecific tRNA-binding domains ( $K_d \sim 0.5 \mu\text{M}$ ). Deletion of the appended domain from the yeast enzyme severely impaired its tRNA binding, aminoacylation, and complementation activities. This N-domain-deleted yeast valyl-tRNA synthetase mutant could be rescued by fusion of the equivalent domain from its human homologue. Moreover, fusion of the N-domain of the yeast enzyme or its human counterpart to *Escherichia coli* glutaminyl-tRNA synthetase enabled the otherwise “inactive” prokaryotic enzyme to function as a yeast enzyme *in vivo*. Different from the native yeast enzyme, which showed different affinities toward mixed tRNA populations, the fusion enzyme exhibited similar binding affinities for all yeast tRNAs. These results not only underscore the significance of nonspecific tRNA binding in aminoacylation, but also provide insights into the mechanism of the formation of aminoacyl-tRNAs.

Aminoacyl-tRNA synthetases are a group of ancient enzymes, each of which catalyzes the attachment of a specific amino acid to its cognate tRNAs. Aminoacyl-tRNAs are then delivered by elongation factor-1 (EF-1)<sup>3</sup> to ribosomes for protein translation. In prokaryotes, there are typically 20 aminoacyl-tRNA synthetases, one for each amino acid (1–4). In eukaryotes, protein synthesis occurs not only in the cytoplasm, but also in organelles, such as mitochondria and chloroplasts (5). Thus, eukaryotes, such as yeast, commonly have two genes that encode distinct sets of proteins for each aminoacylation activity, one localized to the cytoplasm and the other to the mitochondria. Each set aminoacylates the isoaccepting tRNAs

within its respective cell compartment and is sequestered from the isoacceptors confined in other compartments. In most cases, cytoplasmic and mitochondrial synthetase activities are encoded by two distinct nuclear genes, regardless of the cell compartments to which they are confined. However, in some cases, cytoplasmic and mitochondrial forms of a tRNA synthetase with a given amino acid specificity are encoded by the same nuclear gene through alternative initiation of translation, examples of which include *ALAI* (coding for alanyl-tRNA synthetase) (6, 7), *GRS1* (coding for glycyl-tRNA synthetase) (8), *HTS1* (coding for histidyl-tRNA synthetase) (9), and *VAS1* (coding for valyl-tRNA synthetase (ValRS)) (10). Because the isozymes are targeted to different compartments, the two isoforms of ValRS, for example, cannot be substituted for each other *in vivo*. A similar scenario has been observed for genes encoding mitochondrial and cytoplasmic forms of *Arabidopsis thaliana* alanyl-tRNA synthetase, threonyl-tRNA synthetase, and ValRS (11).

Many yeast cytoplasmic tRNA synthetases contain an N- or C-terminal polypeptide extension, which is absent from their bacterial homologs (12). A well-studied example is the appended domain (Ad) of yeast glutaminyl-tRNA synthetase (GlnRS), which binds crude yeast tRNAs, single-stranded RNA, and pseudoknot RNA with comparable affinities; the  $K_d$  values are  $\sim 0.6 \mu\text{M}$  (13, 14). Similar examples have been identified in tRNA synthetases of higher eukaryotes, such as the EMAPII-like domain of plant methionyl-tRNA synthetase (15), the repeat domain of human methionyl-tRNA synthetase (16), and the N-terminal domain of mammalian lysyl-tRNA synthetase (17, 18). In addition to serving as a cis-acting tRNA-binding domain, the Ads of some yeast tRNA synthetases were found to participate in protein-protein interactions, such as those of yeast glutamyl-, methionyl-, (19), and seryl-tRNA synthetases (20). These interactions were shown to enhance tRNA binding and aminoacylation of the associated synthetases (19, 20).

Interestingly, many of the Ads of yeast tRNA synthetases contain one or several canonical nuclear localization signals (21), which are thought to play a role in the nuclear import of these otherwise “cytoplasmic” proteins. Nuclear aminoacylation of tRNAs may serve as a functional checkpoint for the maturity of tRNAs before they are exported from the nucleus (22, 23). By contrast, in higher eukaryotes, nine aminoacyl-tRNA synthetases and three auxiliary proteins (p43, p38, and p18) form a multi-enzyme complex through interactions of their hydrophobic Ads (24). In addition, ValRS from mammalian cells is exclusively isolated as a high molecular mass complex with the elongation factor, EF-1H (25–27). Recently, sev-

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<sup>3</sup> The abbreviations used are: EF-1, elongation factor-1; Ad, appended domain; 5-FOA, 5-fluoroorotic acid; GlnRS, glutaminyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; Wt, wild type; NTA, nitrilotriacetic acid.

## A Cis-acting tRNA-binding Domain

eral tRNA synthetases from prokaryotes as well as eukaryotes have been shown to take part in functions beyond aminoacylation, including roles in mitochondrial RNA splicing, transcriptional and translational regulation, cytokine-like activity, and amino acid biosynthesis (28, 29).

In this report, we focused on the tRNA binding affinity and specificity of the Ad of yeast ValRS. Our results showed that the Ad of yeast ValRS is a cis-acting nonspecific tRNA-binding domain that significantly contributes to the tRNA binding and aminoacylation activity of the native enzyme. Fusion of this N-domain or its human counterpart (a positively charged portion of the Ad of human ValRS) to *Escherichia coli* GlnRS enabled the prokaryotic enzyme to efficiently bind yeast tRNAs and rescue the growth defect of a yeast *GLN4* (encoding GlnRS) knock-out strain. Gel mobility shift assays further showed that this fusion enzyme bound to crude yeast tRNAs without specificity. These results suggest that while the Ad of yeast or human ValRS confers a nonspecific tRNA binding activity to the enzyme, the specificity of tRNA aminoacylation ( $k_{\text{cat}}$  discrimination) was still maintained by the catalytic core of the enzyme.

### EXPERIMENTAL PROCEDURES

**Construction of Various VAS1 Plasmids**—Cloning of a wild-type (Wt) *VAS1* gene into the low-copy-number yeast shuttle vector, pRS315, was previously described (30). A short DNA duplex coding for a FLAG tag was inserted in-frame at the 3'-end of the *VAS1* gene. To facilitate cloning of various internal deletion constructs of *VAS1*, an NdeI site (CATATG) was first introduced by site-directed mutagenesis at the initiator codon of the cytoplasmic form of ValRS. (For convenience, the first amino acid residue of the cytoplasmic form of yeast ValRS is referred to as residue 1.) Subsequently, the DNA sequence coding for amino acid residues 98~1058 of ValRS was PCR-amplified as an NdeI-XhoI fragment and substituted for the NdeI-XhoI fragment of the *VAS1* construct that had an NdeI site inserted at the initiator codon, yielding  $\Delta 1-97$ . To construct  $\Delta 32-71$ , the DNA sequence coding for residues 72~1058 of ValRS was PCR-amplified as a Bsu36I-XhoI fragment and substituted for the Bsu36I-XhoI fragment of the Wt *VAS1* construct. Note that the Wt *VAS1* gene contains a unique Bsu36I cleavage site at nucleotides 91~97 (amino acid residues 31~32) relative to the ATG initiator of the cytoplasmic form.

For fusion of the positively charged portion of the Ad of human ValRS (residues 200~298) to  $\Delta 1-97$ , DNA sequence coding for the portion was PCR-amplified as an NdeI-NdeI fragment and inserted into the NdeI site of  $\Delta 1-97$ , yielding Ad(HsValRS)- $\Delta 1-97$ . For fusion of the Ad of yeast ValRS (residues 1~97) to *E. coli* GlnRS, DNA sequence coding for the Ad was PCR-amplified as an NdeI-NdeI fragment and inserted into the NdeI site at the 5'-end of the open reading frame of *E. coli glnS* cloned in pADH (13). To purify the Wt or truncated versions of yeast ValRS, DNA sequences coding for these enzymes were individually amplified as an NdeI-XhoI fragment and cloned into pADH (with a short DNA sequence coding for a His<sub>6</sub> tag following the XhoI site). The expression of these constructs was under the control of a constitutive *ADH* promoter.

**Complementation Assays for Cytoplasmic Function**—The yeast *VAS1* knock-out strain, CW1, was previously described (30). This strain is maintained by a plasmid containing the Wt *VAS1* gene and a *URA3* marker. Complementation assays for cytoplasmic ValRS activity were carried out by introducing a test plasmid carrying the gene of interest and a *LEU2* marker into CW1 and determined by the ability of the transformants to grow in the presence of 5-fluoroorotate (5-FOA). Starting from a cell density of 4.0  $A_{600}$ , cell cultures were 3-fold serially diluted, and 10- $\mu$ l aliquots of each dilution were spotted onto the designated plates containing 5-FOA. The plates were incubated at 30 °C for 3~5 days. The transformants evicted the maintenance plasmid with a *URA3* marker in the presence of 5-FOA, and thus could not grow on the selection medium unless a functional cytoplasmic ValRS was encoded by the test plasmid. Complementation assays for *E. coli glnS* fusion constructs essentially followed a similar strategy, except that the *GLN4* knock-out strain, EFW6, was used instead (31).

**Complementation Assays for Mitochondrial Function**—CW1 was co-transformed with a test plasmid (carrying a *LEU2* marker) and a second maintenance plasmid (carrying a *HIS3* marker) that expresses only the cytoplasmic form of ValRS (due to a mutation in its ATG1 initiator codon). In the presence of 5-FOA, the first maintenance plasmid (carrying a *URA3* marker) was evicted from the co-transformants, while the second maintenance plasmid was retained. Thus, all co-transformants survived 5-FOA selections, due to the presence of the cytoplasmic ValRS derived from the second maintenance plasmid. The mitochondrial phenotypes of co-transformants were further tested on YPG plates at 30 °C, with results documented on day 3 following plating. Because a yeast cell cannot survive on glycerol without functional mitochondria, the co-transformants did not grow on the YPG plates unless a functional mitochondrial ValRS was generated from the test plasmid.

**Western Blot Analysis**—The protein expression patterns of *VAS1* fusions were determined by a chemiluminescence-based Western blot analysis. INVScl (Invitrogen, Carlsbad, CA) was first transformed with the fusion constructs, and total protein extracts were prepared from each transformant. Aliquots of the protein extracts (40  $\mu$ g) were loaded onto a mini gel containing 10% polyacrylamide (size: 8  $\times$  10 cm) and electrophoresed at 100 V for 1~2 h. Following electrophoresis, the resolved proteins were transferred using a semi-dry transfer device to a polyvinylidene fluoride membrane in buffer containing 30 mM glycine, 48 mM Tris base, pH 8.3, 0.037% sodium dodecylsulfate, and 20% methanol. The membrane was probed with a mouse anti-FLAG tag antibody (Sigma) followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-mouse IgG antibody), and then exposed to x-ray film following the addition of the appropriate substrates. If the proteins of interest were His<sub>6</sub>-tagged, then an HRP-conjugated anti-His<sub>6</sub> tag antibody (Invitrogen) was used instead.

**Purification of His<sub>6</sub>-tagged Proteins**—Purification of the His<sub>6</sub>-tagged proteins followed the protocols provided by the manufacturer of the nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Hilden, Germany) with minor modifications. After breaking the cell, the high-speed supernatant (1 h, 30,000  $\times$  g) was loaded onto a Ni-NTA column equilibrated with 0.1 M

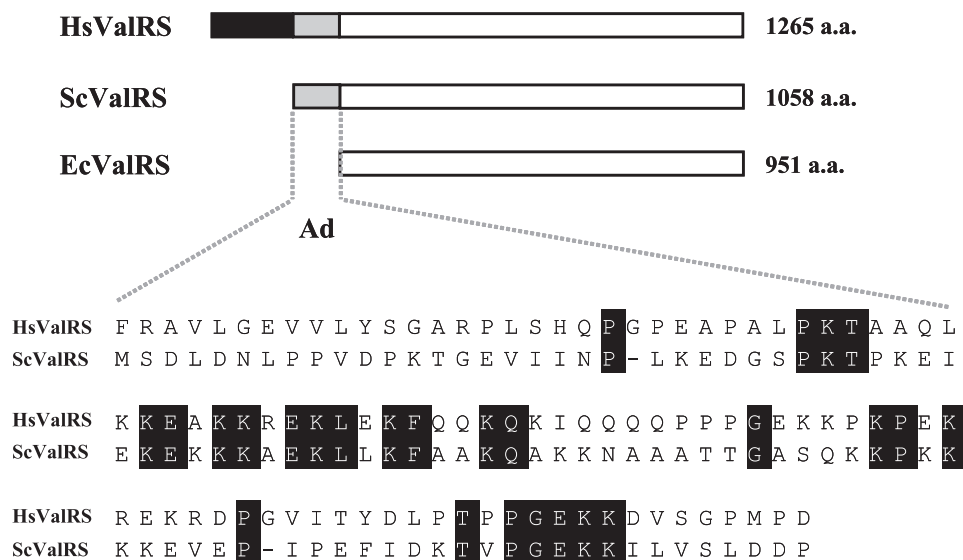


FIGURE 1. **Ads of yeast and human ValRSs.** A, comparison of *E. coli*, yeast, and human ValRSs. Although the catalytic cores of ValRSs from human, yeast, and *E. coli* are significantly homologous to one another, their N termini vary. The yeast protein contains an N-terminal domain of ~97 residues, which is absent from its *E. coli* counterpart, but is conserved in its human homologue. The Ad of human ValRS can be roughly divided into two parts: a hydrophobic portion (residues 1–199) and a positively charged portion (residues 200–298). B, sequence alignment between the Ad of yeast ValRS (residues 1–97) and the positively charged portion of the Ad of human ValRS (residues 200–298). *Hs*, *Homo sapiens*; *Sc*, *S. cerevisiae*; *Ec*, *E. coli*.

sodium phosphate buffer (pH 7.5) containing 300 mM NaCl, 10 mM imidazole, and 10% glycerol (buffer A). The column was washed with ten column volumes of buffer A containing 30 mM imidazole. The protein of interest was then eluted with 0.1 M Tris-HCl, pH 7.5 containing 300 mM NaCl, 10% glycerol, and 300 mM imidazole. Fractions containing the His<sub>6</sub>-tagged protein were pooled, concentrated with polyethylene glycol, and dialyzed against two changes of 0.1 M Tris-HCl, pH 7.5, containing 50 mM NaCl and 40% glycerol.

**Aminoacylation Assay**—Aminoacylation reactions were carried out at ambient temperature in a buffer containing 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 4 mM ATP, 0.1 mg/ml bovine serum albumin, 0.1 mM brewer's yeast tRNA (F. Hoffmann-La Roche, Basel, Switzerland), and 30 μM valine (5 μM [<sup>3</sup>H]valine; Moravik Biochemicals, Brea, CA). The specific activity of [<sup>3</sup>H]valine used was 35.0 Ci/mmol.

The final concentration of ValRS used in the reaction was either 5 or 200 nM. Reactions were quenched by spotting 10-μl aliquots of the reaction mixture onto Whatman filters soaked in 5% trichloroacetic acid and 1 mM valine. The filters were washed three times, for 15 min each, in ice-cold 5% trichloroacetic acid before liquid scintillation counting. Data were obtained from at least three independent experiments and averaged. Kinetic parameters for the Wt and mutant enzymes were determined by directly fitting the data points to the Michaelis-Menten equation. Initial rates of aminoacylation were determined at 25 °C with tRNA<sup>Val</sup> concentrations ranging 0.5–20 μM and enzyme concentrations ranging 15–300 nM. To prepare yeast tRNA<sup>Val</sup> for determination of the kinetic parameters, yeast tRNA<sup>Val</sup> was transcribed using a DNA template constructed from complementary synthetic oligonucleotides containing a 12-bp overlapping region, which were then extended using the Klenow fragment of *E. coli*

DNA polymerase I. Concentrations of tRNAs were determined by absorbance at 260 nm using calculated extinction coefficients based on base compositions.

**Affinity Co-electrophoresis**—Protein-tRNA interactions were assayed using affinity co-electrophoresis as previously described (13, 32). Crude yeast tRNA or *in vitro*-transcribed yeast tRNA<sup>Val</sup> was labeled with <sup>32</sup>P using polynucleotide kinase (New England Biolabs, Beverly, MA), after dephosphorylation with calf intestine phosphatase. Proteins to be tested were 2-fold serially diluted and mixed with a 5% polyacrylamide solution, forming a mini-gel matrix with a protein gradient of 0.09–24 or 0.003–0.8 μM. Labeled tRNA was loaded into each well at an estimated concentration of 1 nM in 2-μl aliquots. Gels were run in a buffer containing 1× TBE (90 mM

Tris borate and 2 mM EDTA) and 50 mM NaCl at 20 °C at 50 V for 1 h. After electrophoresis, gels were vacuum-dried and analyzed with a PhosphorImager (Bio-Rad). Dissociation constants (*K<sub>d</sub>*) of the proteins and tRNA were determined as previously described (33).

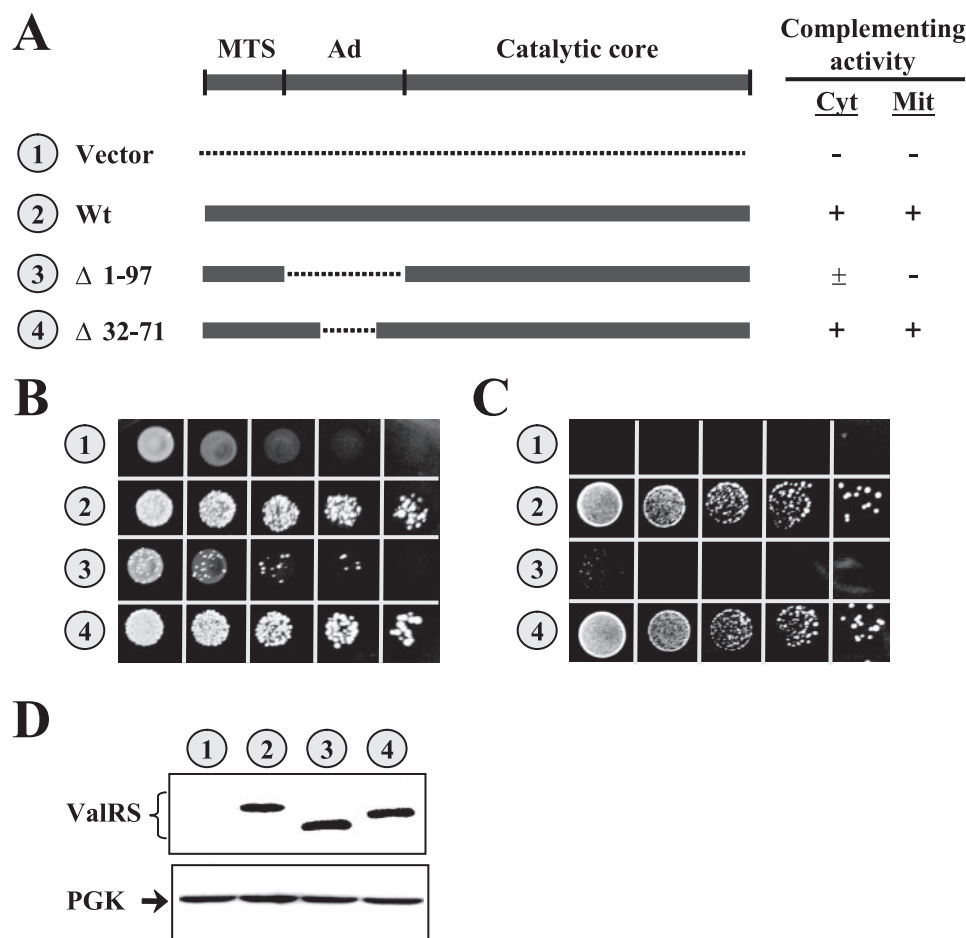
## RESULTS

**The Ad of Yeast ValRS Is Important for *in Vivo* Complementation Activity**—Previous studies showed that a single *VAS1* gene specifies both the mitochondrial and cytoplasmic forms of ValRS through alternative initiation of translation from two in-frame AUG codons 138 nucleotides apart (10, 30). Hence, the mitochondrial precursor form carries the same polypeptide sequence as its cytoplasmic counterpart, except for an N-terminal mitochondrial targeting sequence that is subsequently cleaved away upon being imported into mitochondria by a matrix-processing peptidase. (For convenience, the first amino acid residue of the cytoplasmic form of yeast ValRS is referred to as residue 1.) Comparison of *E. coli*, yeast (the cytoplasmic form), and human ValRSs showed that the catalytic cores of these enzymes are significantly homologous to one another (~30% identity), but their N termini vary (Fig. 1). The yeast enzyme has a polypeptide extension of ~97 residues, which is absent from its *E. coli* counterpart. Embedded inside this appended domain (Ad) is a lysine-rich cluster of 40 residues (residues 32–71) that contains several canonical nuclear localization signals (21). These signals are thought to take part in the nuclear localization of this otherwise “cytoplasmic” enzyme.

In contrast to the yeast enzyme, human ValRS has acquired an additional hydrophobic domain (residues 1–199) that is responsible for interacting with the elongation factor, EF-1H (25, 26, 34), while conserving the positively charged domain (residues 200–298) that distinguishes the yeast enzyme from its bacterial counterparts (Fig. 1). The primary sequences of the



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**FIGURE 2. Complementation assays for Wt and mutant yeast VAS1 constructs.** CW1, a yeast VAS1 knock-out strain, was transformed with Wt and mutant VAS1 constructs, and the ability of the transformants to rescue the growth defect of the knock-out strain was tested. *A*, schematic summary of VAS1 constructs and their complementation activities. Shaded boxes and dashed lines denote sequences coding for yeast ValRS and deletions, respectively. The symbols “+” and “-” indicate positive and negative complementation, respectively. *Mit*, mitochondrial; *Cyt*, cytoplasmic. *B*, complementation assays for cytoplasmic ValRS activity on a 5-FOA plate. *C*, complementation assays for mitochondrial ValRS activity on a YPG plate. *D*, assay of protein expression by Western blotting. Upper panel, ValRS; lower panel, PGK (as a loading control). Numbers 1–4 (circled) in *B* and *C* represent the constructs shown in *A*.

catalytic cores of the yeast and human enzymes share ~50% identity in primary sequence. It is worth mentioning that while the Ad of yeast ValRS and the equivalent domain in human ValRS are both rich in lysine residues (16 and 22%, respectively) and have similar molecular sizes (97 and 99 residues, respectively), the primary sequences share only 26% identity. So far, little is known about the biological function of the Ad of yeast ValRS or its counterpart in the human enzyme.

To investigate whether the Ad is essential for the *in vivo* function of yeast ValRS, various segments were deleted from the Ad of the Wt VAS1 sequence, and the ability of the resultant constructs to restore the growth phenotype of a *vas1*<sup>-</sup> yeast strain on 5-FOA and YPG was tested. As summarized in Fig. 2*A*, deletion of the lysine-rich insert (residues 32~71) from the Ad had little effect on complementation activity; the truncated enzyme, Δ32–71, effectively rescued the growth defect of the knock-out strain on 5-FOA and YPG (Fig. 2, *B* and *C*, number 4). However, deletion of the entire Ad (residues 1~97) severely impaired the complementation activity; the truncated enzyme, Δ1–97, showed poor cytoplasmic activity and little mitochon-

drial activity (Fig. 2, *B* and *C*, number 3). To examine whether the defective phenotype of Δ1–97 was caused by severe protein degradation, we next compared the protein expression levels of these constructs by Western blotting using an anti-FLAG tag antibody. As shown in Fig. 2*D*, a similar level of protein was detected in Wt, Δ32–71, and Δ1–97, suggesting that the defective phenotype of Δ1–97 was not caused by lower levels of protein expression.

*The Ad of Yeast ValRS Is Critical for in Vitro Aminoacylation Activity*—To investigate whether these deletion constructs retain aminoacylation activity *in vitro*, His<sub>6</sub>-tagged ValRS proteins were purified to homogeneity using Ni-NTA column chromatography and were assayed using unfractionated yeast tRNA as the substrate. Aminoacylation was carried out at a final concentration of 5 nM ValRS protein. As shown in Fig. 3*A*, Δ32–71 and Δ1–97 had little aminoacylation activity *in vitro* compared with the Wt under the conditions used. To get a better idea of the possible difference in aminoacylation activity between Δ32–71 and Δ1–97, the protein concentration used for the assay was increased by as much as 40-fold. As shown in Fig. 3*B*, Δ32–71 showed significantly higher aminoacylation activity than did

Δ1–97, which might explain why Δ32–71 retained a near Wt growth phenotype *in vivo* and Δ1–97 did not (Fig. 2). Kinetic parameters for these enzymes were subsequently determined using *in vitro*-transcribed yeast tRNA<sup>Val</sup> as the substrate. As shown in Table 1, the  $K_m$  and  $k_{cat}$  values for the Wt were 0.2 μM and 0.2 s<sup>-1</sup>, respectively, while the  $K_m$  and  $k_{cat}$  values for Δ32–71 were 10 μM and 0.06 s<sup>-1</sup>, respectively, under the conditions described under “Experimental Procedures.” So, deletion of the lysine-rich insert drastically reduced the enzyme affinity for tRNA<sup>Val</sup> (a 50-fold reduction), but only slightly reduced its turnover number (a 3-fold reduction). Because Δ1–97 was essentially inactive in aminoacylation, we could not successfully determine its  $K_m$  and  $k_{cat}$  values. Taken together, these results suggested that the lysine-rich insert and the Ad is critical for the tRNA binding of the enzyme.

*The Ad per se Is a Nonspecific tRNA-binding Domain*—Because the Ad is rich in lysine residues (~22%) and important for tRNA binding of the enzyme (Figs. 1 and 2), we wondered whether the Ad itself can bind tRNA and with what affinity. Pursuant to this objective, purified recombinant Ad was sub-

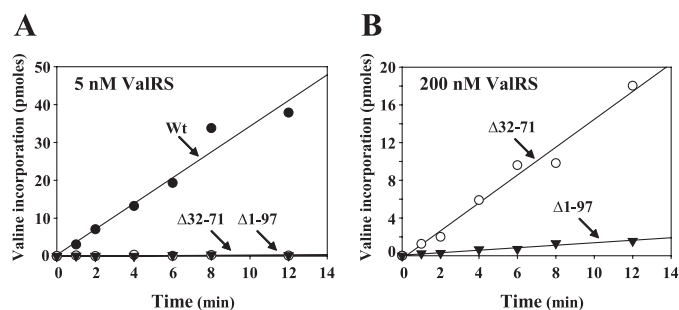


FIGURE 3. **Aminoacylation assays for Wt and mutant yeast ValRSs.** *A*, aminoacylation assay. The aminoacylation activity of the Wt and mutant ValRSs was tested at a final concentration of 5 nM. The relative amounts of [<sup>3</sup>H]valine that were incorporated into tRNA were subsequently determined by a liquid scintillation counter. *B*, aminoacylation assay. The aminoacylation activity of the mutant ValRSs was tested at a final concentration of 200 nM.

TABLE 1

Kinetic parameters for aminoacylation of tRNA<sup>Val</sup> by wild-type and mutant ValRS

ValRS variant	$K_m$ $\mu\text{M}$	$k_{\text{cat}}$ $\text{s}^{-1}$	$k_{\text{cat}}/K_m$ $\text{M}^{-1}\text{s}^{-1}$	Relative efficiency
Wild-type	$0.2 \pm 0.02^a$	$0.2 \pm 0.04$	$1 \times 10^6$	1
$\Delta 32-71$	$10 \pm 0.08$	$0.06 \pm 0.02$	$6 \times 10^3$	0.006
$\Delta 1-97$	ND <sup>b</sup>	ND	ND	ND

<sup>a</sup> Each value is determined from a hyperbolic fit of two independent data sets.

<sup>b</sup> ND, not determined.

jected to a gel mobility-shift assay, also known as polyacrylamide gel coelectrophoresis. An aliquot of <sup>32</sup>P-labeled *in vitro*-transcribed yeast tRNA<sup>Val</sup> or unfractionated yeast tRNA (~1 nM) was loaded into each well of a 5% polyacrylamide gel, where 2-fold dilutions of the purified Ad had been incorporated into the gel, forming a protein gradient of 0.09~24  $\mu\text{M}$ . No protein was incorporated into the left-most lane as a control. As shown in Fig. 4, the Ad had a  $K_d$  of 2.0 and 0.5  $\mu\text{M}$  toward unfractionated yeast tRNA and *in vitro*-transcribed yeast tRNA<sup>Val</sup>, respectively (Fig. 4, *A* and *B*), suggesting that the Ad *per se* is a nonspecific tRNA-binding domain.

To advance understanding of the functional role of the Ad in the tRNA binding of the native enzyme, the  $K_d$  values of the Wt and truncated enzymes ( $\Delta 32-71$  and  $\Delta 1-97$ ) for tRNA<sup>Val</sup> were also examined using a similar approach. As shown in Fig. 4,  $\Delta 1-97$  and  $\Delta 32-71$  lost much of their tRNA binding activity (with a  $K_d > 24 \mu\text{M}$  in both cases) compared with the Wt (with a  $K_d$  of ~0.05  $\mu\text{M}$  for tRNA<sup>Val</sup>) (Fig. 4, *D-F*). This result suggests that the lysine-rich insert and the Ad significantly contribute to the tRNA binding activity of the native enzyme, which is consistent with the kinetic parameters shown in Table 1. In addition, it was interesting to find that the native enzyme has an apparent affinity for tRNA<sup>Val</sup>, which was ~10-fold higher than that of the Ad (compare Fig. 4, *B* and *D*) and more than 50-fold higher than that of the catalytic core (compare Fig. 4, *D* and *E*), suggesting that the Ad may act synergistically with the catalytic core to form a high-affinity binding site for tRNA<sup>Val</sup>. Furthermore, different from the tRNA binding characteristics of the Ad, which showed similar binding affinities for mixed tRNA populations, the native enzyme exhibited heterogeneous binding curves toward crude yeast tRNA (compare Fig. 4, *A* and *C*). Because crude yeast tRNA contains many different tRNA species, it is not clear whether the tRNA population that bound the

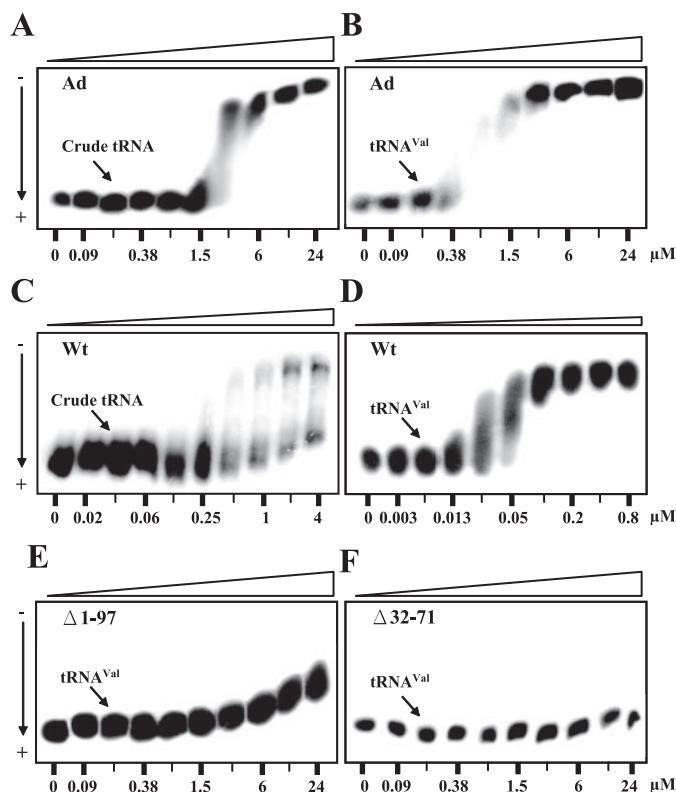
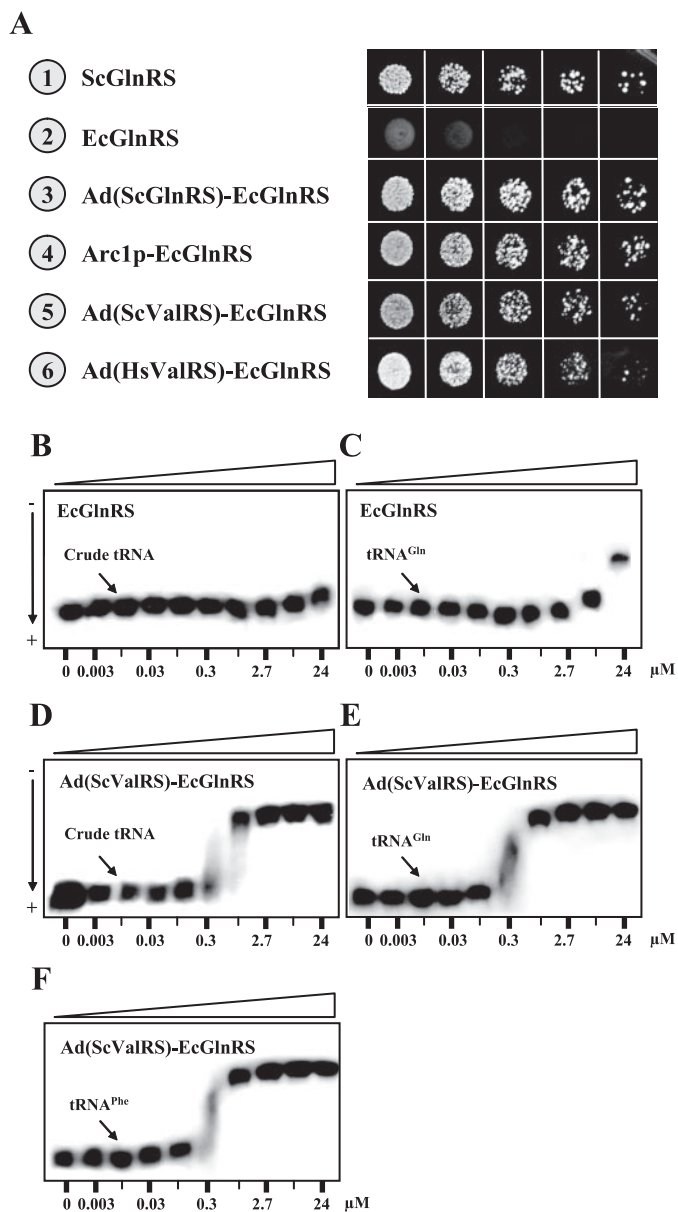


FIGURE 4. **Gel mobility-shift assays for the tRNA binding activities of the Ad and Wt and mutant yeast ValRSs.** Affinities of the Ad and Wt and mutant ValRSs toward <sup>32</sup>P-labeled unfractionated yeast tRNA or *in vitro*-transcribed yeast tRNA<sup>Val</sup> were determined by polyacrylamide gel coelectrophoresis. At the bottom of each gel are the concentrations of the protein used. As a control, the left-most lane in each gel contained no protein. *A*, Ad binding unfractionated yeast tRNA. *B*, Ad binding yeast tRNA<sup>Val</sup>. *C*, Wt binding unfractionated yeast tRNA. *D*, Wt binding yeast tRNA<sup>Val</sup>. *E*,  $\Delta 1-97$  binding yeast tRNA<sup>Val</sup>. *F*,  $\Delta 32-71$  binding yeast tRNA<sup>Val</sup>.

yeast enzyme with higher affinity represents only the cognate tRNAs (Fig. 4C).

*The Ad of Yeast ValRS Converted E. coli GlnRS into a Functional Yeast Enzyme*—We previously showed that *E. coli* GlnRS could not charge yeast tRNA and therefore was unable to rescue the growth defect of EFW6, a yeast *GLN4* (encoding GlnRS) knock-out strain, but fusion of Arc1p or the Ad of yeast GlnRS to the *E. coli* enzyme enabled the fusion enzyme to charge yeast tRNA and restore the growth phenotype of the knock-out strain (13, 31) (Fig. 5). Because the Ad of yeast ValRS possesses a similar nonspecific tRNA binding activity, we wondered whether this domain could also rescue the *E. coli* enzyme. To test this possibility, the Ad of yeast ValRS was fused in-frame to the N terminus of *E. coli* GlnRS (resulting in Ad(ScValRS)-EcGlnRS), and the complementation activity of the fusion construct was tested. As shown in Fig. 5, Ad(ScValRS)-EcGlnRS successfully restored the growth phenotype of EFW6 on 5-FOA (Fig. 5A, number 5). Given that aminoacylation generally takes place under conditions wherein an active synthetase/tRNA complex has been formed, this effect of reviving an “inactive” enzyme with a nonspecific tRNA-binding domain was particularly remarkable. Perhaps, the Ad can increase the affinity of the fusion enzyme for all tRNAs, including yeast tRNA<sup>Gln</sup>, and this elevated affinity for yeast tRNA<sup>Gln</sup> might be sufficient for the

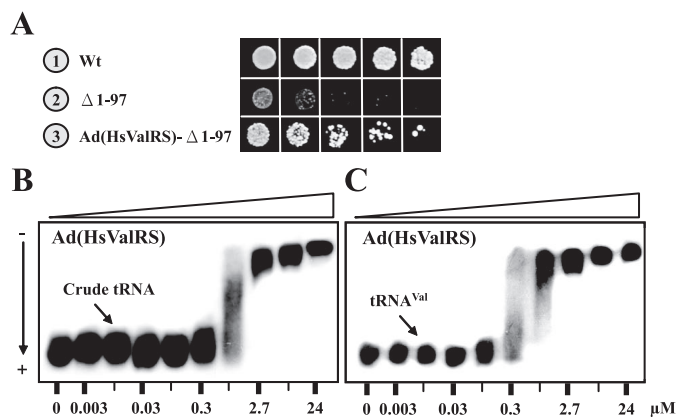
## A Cis-acting tRNA-binding Domain



**FIGURE 5. Converting *E. coli* GlnRS into a functional yeast enzyme.** Arc1p and the Ads of yeast GlnRS, yeast ValRS, and human ValRS were independently fused at the N terminus of *E. coli* GlnRS, and the ability of the fusion enzymes to rescue the growth defect of a yeast *GLN4* knock-out strain was tested. *A*, complementation assays for cytoplasmic GlnRS activity on a 5-FOA plate. *B*, *E. coli* GlnRS binding unfractionated yeast tRNA. *C*, *E. coli* GlnRS binding yeast tRNA<sup>Gln</sup>. *D*, Ad(ScValRS)-EcGlnRS binding unfractionated yeast tRNA. *E*, Ad(ScValRS)-EcGlnRS binding yeast tRNA<sup>Gln</sup>. *F*, Ad(ScValRS)-EcGlnRS binding yeast tRNA<sup>Phe</sup>.

fusion enzyme to confer the necessary aminoacylation activity to the knock-out strain.

To gain further insights into the tRNA binding properties of the fusion enzyme, the apparent binding affinities of the fusion enzyme for yeast tRNA<sup>Gln</sup> and unfractionated yeast tRNA were determined. As shown in Fig. 5, *E. coli* GlnRS did not significantly bind unfractionated yeast tRNA or yeast tRNA<sup>Gln</sup> (with a  $K_d > 24 \mu\text{M}$  in both cases) (Fig. 5, *B* and *C*). However, upon fusion of the *E. coli* enzyme with the Ad of yeast ValRS, the affinities of the fusion enzyme for yeast tRNA<sup>Gln</sup>, tRNA<sup>Phe</sup>, and unfractionated yeast tRNA were drastically increased. The



**FIGURE 6. Rescuing a defective yeast ValRS mutant with the Ad of human ValRS.** The positively charged portion of the Ad of human ValRS (residues 200–298) was fused at the N terminus of the truncated yeast ValRS mutant,  $\Delta 1-97$ , and the ability of the fusion protein to rescue the growth defect of a yeast *VAS1* knock-out strain was tested. *A*, positively charged portion of the Ad of human ValRS (residues 200–298) was fused at the N terminus of the truncated yeast ValRS mutant,  $\Delta 1-97$ , and the ability of the fusion protein to rescue the growth defect of a yeast *VAS1* knock-out strain was tested. *B*, Ad of human ValRS (residues 200–298) binding unfractionated yeast tRNA. *C*, Ad of human ValRS (residues 200–298) binding yeast tRNA<sup>Val</sup>.

fusion enzyme bound these tRNA ligands with comparable affinities, at  $K_d$  values of  $\sim 0.5 \mu\text{M}$  (Fig. 5, *D–F*). Thus, the Ad enhanced the binding affinity of the prokaryotic enzyme for cognate and noncognate tRNAs. While our previous reports demonstrated that the Ad of yeast GlnRS and Arc1p increased the binding affinity of *E. coli* GlnRS for yeast tRNA<sup>Gln</sup> (13, 14), this appears to be the first piece of experimental evidence showing that the Ad increases the binding affinity of the prokaryotic enzyme not only to tRNA<sup>Gln</sup>, but also to noncognate tRNAs.

In contrast to the yeast enzyme, human ValRS has a rather large Ad (residues 1–298). The N-terminal part of the Ad (residues 1–199) is hydrophobic in nature and is responsible for interacting with the elongation factor, EF-1H, while the C-terminal part (residues 200–298) is positively charged and is thought to be homologous to the Ad of yeast ValRS. It should be mentioned, however, that the primary sequence of the positively charged portion of the Ad of human ValRS actually shares only 26% identity with that of the Ad of yeast ValRS (Fig. 1). Remarkably, fusion of the lysine-rich domain of human ValRS (residues 200–298) to *E. coli* GlnRS also rescued the prokaryotic enzyme; the fusion enzyme Ad(HsValRS)-EcGlnRS successfully restored the growth phenotype of the *GLN4* knock-out strain on 5-FOA (Fig. 5*A*, number 6), reinforcing the notion that this portion of human ValRS could be a nonspecific tRNA-binding domain.

*The Defective Yeast ValRS Mutant,  $\Delta 1-97$ , Was Rescued by the Ad of Human ValRS*—To further elucidate the tRNA binding property of the lysine-rich domain of human ValRS (residues 200–298), we next fused this domain to the yeast ValRS mutant  $\Delta 1-97$ , which was shown to be defective in tRNA binding, aminoacylation, and complementation (Figs. 2–4), and the complementation activity of the resultant construct was tested. As shown in Fig. 6*A*, fusion of the lysine-rich domain of human ValRS to  $\Delta 1-97$ , resulting in Ad(HsValRS)- $\Delta 1-97$ , significantly improved the complementation activity of the truncated yeast enzyme; Ad(HsValRS)- $\Delta 1-97$  successfully rescued the growth



defect of the *VAS1* knock-out strain on 5-FOA. Since the hydrophobic portion of the Ad of human ValRS was previously assigned a role of interacting with EF-1H, the notion that the adjacent domain possesses nonspecific tRNA binding activity was particularly noteworthy in terms of tRNA turnover. To directly test the tRNA binding activity of the lysine-rich domain, the DNA sequence coding for residues 200–298 of human ValRS was cloned and expressed, and the recombinant protein was purified to homogeneity using Ni-NTA column chromatography. Fig. 6 shows that this domain bound yeast tRNA<sup>Val</sup> and unfractionated yeast tRNA with comparable affinity, the  $K_d$  values of which were  $\sim 0.5 \mu\text{M}$  (Fig. 6, B and C). So, the nonspecific tRNA binding activity has been conserved in the Ads of yeast and human ValRSs during evolution.

## DISCUSSION

A general feature regarding the Ads of yeast tRNA synthetases is their intrinsic capability to nonspecifically bind to RNA (12). Such activity is thought to enhance the overall efficiency of aminoacylation by attracting tRNA to the vicinity of the catalytic core. The binding affinity of a tRNA synthetase toward its cognate tRNAs is generally characterized by dissociation constants of the order of  $0.1\text{--}1 \mu\text{M}$  under physiological conditions (35). The relatively low affinity ensures that the synthetases (or tRNAs) turn over rapidly during aminoacylation and translation. Interestingly, the binding affinities of the Ads of yeast and human ValRSs toward tRNA<sup>Val</sup> also fall into this range (Figs. 4, 6), making them useful as a tRNA-binding cofactor during aminoacylation.

As with the Ad of yeast GlnRS, for which the tRNA binding activity is mainly attributed to two lysine-rich clusters located at both ends of the Ad (14), the tRNA binding activity of the Ad of yeast ValRS appears to be largely attributable to a positively charged sequence between residues 32 and 71. However, unlike the Ad of yeast GlnRS, where large deletions in the Ad had little effect on the *in vitro* aminoacylation activity (36) or *in vivo* complementation activity (37), the Ad of yeast ValRS played a critical role in the biochemical activity of the enzyme. Deletion of just the lysine-rich cluster from the valine enzyme severely impaired its tRNA binding and aminoacylation activities ( $\Delta 32\text{--}71$  in Figs. 3 and 4). Kinetic studies further showed that the tRNA binding affinity ( $K_m$ ) and catalytic efficiency ( $k_{\text{cat}}/K_m$ ) of this truncated enzyme were reduced by more than 50-fold compared with those of the Wt (Table 1). However, despite the drastic impairment in activity, this truncated enzyme still retained its complementing activity *in vivo* ( $\Delta 32\text{--}71$  in Fig. 2). Therefore, it appears that a mutant enzyme with as low as 1%–2% aminoacylation activity relative to the native enzyme is sufficient to retain a near Wt growth phenotype for the knock-out strain. Perhaps for a similar reason, fusion of the Ads of yeast and human ValRSs to *E. coli* GlnRS enabled this prokaryotic enzyme to rescue the growth defect of the yeast knock-out strain EFW6 (Fig. 5). This finding was particularly inspiring in view of the fact that while these Ads possess only nonspecific tRNA binding activity, they can promote the formation by the prokaryotic enzyme of an active complex with yeast tRNA<sup>Gln</sup> and not an abortive complex that is often seen with non-cog-

nate or mutant tRNAs. This also reinforces the notion that  $k_{\text{cat}}$  discrimination plays a key role in the catalysis and formation of Gln-tRNA<sup>Gln</sup>.

In contrast to the nonspecific tRNA binding activity of the Ads and the fusion protein, Ad(ScValRS)-EcGlnRS, native yeast ValRS appeared to possess certain tRNA specificity (Figs. 4–6). It is likely that the Ad and the catalytic core of the yeast enzyme have coevolved during evolution to form a high-affinity binding site for tRNA<sup>Val</sup>. Therefore, the native yeast enzyme had higher affinity for tRNA<sup>Val</sup> than did the Ad or the catalytic core alone (Fig. 4, B, D, E). So far, it is not clear whether the yeast enzyme only binds cognate tRNAs with high affinity. A comparative binding assay with various purified isoacceptors is currently underway to resolve this issue. Although the Ads of GluRS and MetRS are also rich in positively charged residues and important for aminoacylation, they do not function as tRNA-binding domains. Instead, these Ads specifically interact with a tRNA-binding cofactor, Arc1p, which, in turn, recruits tRNA to the associated enzymes for aminoacylation (38). A functionally similar tRNA-recruiting domain was identified in an auxiliary protein associated with the mammalian multi-synthetase complex (39).

ValRS from mammalian cells is exclusively isolated as a high molecular mass complex with the elongation factor, EF-1H (25–27). Like yeast ValRS, the mammalian enzyme also contains a strong affinity for the polyanionic carrier heparin-Ultrogel. However, the mammalian enzyme exhibits additional hydrophobic properties (34). Sequence analysis revealed that mammalian ValRS has conserved the positively charged N-terminal extension (residues 200–298) that distinguishes yeast ValRS from its bacterial counterparts, while acquiring an additional hydrophobic domain (residues 1–199) that is responsible for interacting with the four subunits of elongation factor EF-1H ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  subunits) (25–27, 40) (Fig. 1). Excision of the hydrophobic extension (residues 1–200) by the enzyme, elastase, severely impaired association with EF-1H, but had little effect on the *in vitro* catalytic activity of the enzyme (40). A recent report further showed that association of mammalian ValRS with EF-1H is required for stimulation of its aminoacylation activity *in-trans* by EF-1 $\alpha$  (41). This observation provides a structural basis for the functional interaction between ValRS and EF-1 $\alpha$ , which may play a role in tRNA channeling by directly transferring valyl-tRNA from the synthetase to ribosomes. In contrast, yeast appears to lack a similar mechanism for tRNA<sup>Val</sup> channeling.

As for the positively charged portion of the Ad of human ValRS (residues 200–298), our results suggested that it possesses a tRNA binding property similar to that of the Ad of yeast ValRS and may act as a tRNA-binding cofactor *in cis* to the catalytic core of the synthetase (Fig. 6). If that is the case, the Ad of human ValRS can concurrently interact with EF-1H (using its hydrophobic sequence) and tRNA (using its positively charged sequence), and further enhance the efficiency of tRNA<sup>Val</sup> channeling. To our knowledge, mammalian ValRS is the only tRNA synthetase that carries such a unique sequence feature. In addition, ValRS may be one of the few examples wherein nonspecific tRNA binding activity is conserved in the Ads of homologous yeast and human

## A Cis-acting tRNA-binding Domain

tRNA synthetases. However, regardless of the detailed interpretation, the most significant findings reported here are the faithful transfer of nonspecific tRNA binding activity from an Ad to a fusion enzyme and the capability of a nonspecific tRNA-binding domain to enhance the formation and catalysis of an active synthetase/tRNA complex. Thus, acquiring a nonspecific tRNA-binding domain might be one of the feasible mechanisms that enables a bacterial tRNA synthetase to gain extra affinity toward yeast tRNA and consequently evolve into a functional yeast enzyme.

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