Schizosaccharomyces pombe Possesses Two Paralogous Valyl-tRNA Synthetase Genes of Mitochondrial Origin

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Abstract

Previous studies showed that VAS1 of Saccharomyces cerevisiae encodes both cytosolic and mitochondrial forms of valyl-tRNA synthetase (ValRS) through alternative initiation of translation. We show herein that except for Schizosaccharomyces pombe, all yeast species studied contained a single ValRS gene encoding both forms, and all of the mature protein forms deduced from those genes possessed an N-terminal appended domain (Ad) that was absent from their bacterial relatives. In contrast, S. pombe contained two distinct nuclear ValRS genes, one encoding the mitochondrial form and the other its cytosolic counterpart. Although the cytosolic form closely resembles other yeast ValRS sequences (~60% identity), the mitochondrial form exhibits significant divergence from others (~35% identity). Both genes are active and essential for the survival of the yeast. Most conspicuously, the mitochondrial form lacks the characteristic Ad. A phylogenetic analysis further suggested that both forms of S. pombe ValRSs are of mitochondrial origin, and the mitochondrial form is ancestral to the cytoplasmic form.

Key words: aminoacylation, appended domain, mitochondrial localization, tRNA binding, valyl-tRNA synthetase.

Introduction

Aminoacyl-tRNA synthetases (aaRSs) are a group of primordial enzymes responsible for protein translation, each of which catalyzes the attachment of a specific amino acid to its cognate tRNAs, forming aminoacyl-tRNAs. These charged tRNAs are then delivered by elongation factor-1 to ribosomes for protein translation. In prokaryotes, there are typically 20 aaRSs, one for each amino acid (Martinis and Schimmel 1991; Carter 1993; Giege et al. 1998; Pelchat and Lapointe 1999). In eukaryotes, protein synthesis occurs not only in the cytoplasm but also in organelles, such as mitochondria and chloroplasts (Dietrich et al. 1992). Thus, eukaryotes, such as yeast, commonly have two genes that encode distinct sets of proteins for each aminoacylation activity, one localized in the cytoplasm and the other in the mitochondria. Each set aminoacylates the isoaccepting tRNAs within its respective cell compartment and is sequestered from the isoaccepters confined to 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 specific to a given amino acid are encoded by the same nuclear gene through alternative initiation of translation, examples of which include ALA1 (which codes for alanyl-tRNA synthetase) (Tang et al. 2004; Chang et al. 2006), GRS1 (which codes for glycyl-tRNA synthetase) (Chang and Wang 2004), HTS1 (which codes for histidyl-tRNA synthetase) (Natsoulis et al. 1986), and VAS1 (which codes for valyl-tRNA synthetase) (Chatton et al. 1988). Because the isozymes target different compartments, the two isoforms of ValRS, for example, cannot be substituted for each other in vivo. A similar scenario was observed for genes encoding mitochondrial and cytoplasmic forms of Arabidopsis thaliana alanyl-tRNA synthetase, threonyl-tRNA synthetase, and ValRS (Souciet et al. 1999).

Many yeast cytoplasmic tRNA synthetases contain an amino- or carboxyl-terminal polypeptide extension that is absent from their bacterial homologs (Mirande 1991) and is believed to have been added to the enzymes later during evolution. 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 ~0.6 µM (Wang and Schimmel 1999; Wang et al. 2000). Similar examples were reported in yeast ValRS (Chang et al. 2008) and tRNA synthetases of higher eukaryotes, such as the EMAPIII-like domain of plant methionyl-tRNA synthetase (Kaminska et al. 2000), the repeat domain of human methionyl-tRNA synthetase (Kaminska et al. 2001), and the amino-terminal domain of mammalian lysyl-tRNA synthetase (Francin et al. 2002; Francin and Miranda 2006). Although these Ads are not part of the characteristic tRNA-binding domain formed primarily by the N-terminal acceptor stem-binding domain and the C-terminal anticodon-binding domain, they are thought to provide an extra tRNA-binding affinity to the catalytic body and help recruit tRNA to the respective enzymes. 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-, and seryl-tRNA synthetases (Godinic et al. 2007). These interactions were shown to enhance their tRNA binding and aminoacylation (Simos et al. 1996; Godinic et al. 2007).
Cloning of the wild-type (WT) and mutant Construction of Various Materials and Methods from an ancestral mitochondrion. The mitochondrial form may represent a primitive form of the yeast enzyme possesses an N-terminal polypeptide extension of ~98 residues that is absent from its bacterial relative and acts “in cis” as a nonspecific tRNA-binding domain (Chang et al. 2008). In this report, we investigated the function and phylogeny of ValRS from 14 different yeast species. It is our hope that results obtained from this study not only advance understanding of the mechanism that enables a single gene to encode two functionally exclusive protein isoforms in particular but also provide new insights into the evolution of eukaryotic ValRSs in general. Our results showed that two distinct ValRS genes exist in Schizosaccharomyces pombe: one specifying the cytoplasmic form and the other its mitochondrial counterpart. Although these two forms significantly diverge from each other, the phylogeny strongly argues that both forms should be regarded as being of mitochondrial origin, and the mitochondrial form may represent a primitive form of eukaryotic ValRS that was transferred to the nucleus from an ancestral mitochondrion.

Materials and Methods

Construction of Various VAS1 Plasmids—Cloning of the wild-type (WT) and mutant VAS1 genes of Saccharomyces cerevisiae was previously described (Wang et al. 2003). To clone VAS1 of Yarrowia lipolytica (YIVAS1) into pADH-2xFLAG (a high-copy-number yeast shuttle vector with a LEU2 marker, a constitutive ADH promoter preceding the multiple cloning sites, and a 2xFLAG sequence following the multiple cloning sites) (Wang et al. 2003), a pair of oligonucleotides was used as primers to amplify the gene via a polymerase chain reaction (PCR) using yeast genomic DNA as the template. The forward primer with an Eagl site is located 96-bp upstream of the first ATG initiator codon of the VAS1 open reading frame, whereas the reverse primer with an Spel site is located immediately upstream of the stop codon. The ~3.3-kb PCR-amplified fragment was subsequently digested with Eagl and Spel prior to cloning into pADH for expression. Cloning of VAS1 of Candida albicans (CaVAS1) into pRS315 (a low-copy-number yeast shuttle vector carrying a LEU2 marker) followed a similar protocol, except that the forward primer was annealed to a sequence ~300-bp upstream of the first ATG initiator codon. Cloning of VAS1 and VAS2 of S. pombe (SpVAS1 and SpVAS2) also followed a similar approach but used reverse-transcribed complementary (c)DNA as the template for PCR amplification. Construction of initiator mutants for these VAS1 genes followed a strategy described earlier (Chang and Wang 2004).

To fuse the predicted mitochondrial targeting sequence of SpValRS2 to ScValRS (the cytoplasmic form of S. cerevisiae ValRS), the DNA sequence containing base pairs −48 to −93 of SpVAS2 was PCR amplified as an Eagl-Xbal fragment and cloned in-frame into the 5’ end of ScVAS1c (coding for the cytoplasmic form of ScValRS), resulting in pRIC89. Fusion of the mitochondrial targeting signal of the mitochondrial form of ScValRS to SpValRS1 followed a similar approach. To check for protein expression and localization of SpValRS1 and SpValRS2 in S. pombe, DNA sequences coding for a 13x-myc tag and green fluorescence protein (GFP) were independently inserted at the 3’ ends of these two genes in the yeast chromosomes by homologous recombination as previously described (Bahler et al. 1998). Construction of S. pombe knockout strains essentially followed the protocol described in Bahler et al. (1998).

Complementation Assays for Cytoplasmic Function

The yeast VAS1 knockout strain, CW1 (MATa, his3Δ1, leu2Δ10, met15Δ10, ura3Δ10, and vas1Δ1), was previously described (Wang et al. 2003). 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 an LEU2 marker into CW1, and the ability of the transformants to grow in the presence of 5-fluoroorotic acid (5-FOA) was determined. Starting from a cell density of 4.0 A600, cell cultures were 5-fold serially diluted, and 20-μl aliquots of each dilution were spotted onto the designated plates containing 5-FOA. 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 Mitochondrial Function

CW1 was cotransformed 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 the ATG1 initiator codon). In the presence of 5-FOA, the first maintenance plasmid (carrying a URA3 marker) was evicted from the cotransformants, whereas the second maintenance plasmid was retained. Thus, all cotransformants survived 5-FOA selections, due to the presence of the cytoplasmic ValRS derived from the second maintenance plasmid. The mitochondrial phenotypes of the cotransformants were further tested on yeast extract peptone glycerol (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 cotransformants 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 the constructs used in the complementation assays were determined by a chemiluminescence-based Western blot analysis. INVSc1 (MATa, his3Δ1, leu2, trp1-289, ura3-52; MATα, his3Δ1, leu2, trp1-289, ura3-52) (Invitrogen, Carlsbad, CA) was first transformed with the constructs of interest, and total
protein extracts were prepared from each transformant. Aliquots of the protein extracts (40 μg) were loaded onto a mini gel (size: 8 × 10 cm) containing 10% polyacrylamide and electrophoresed at 100 V for 1–2 h. Following electrophoresis, the resolved proteins were transferred using a semidy transfer device to a polyvinylidene fluoride membrane in a buffer containing 30 mM glycine, 48 mM Tris base (pH 8.3), 0.037% sodium dodecysulfate, and 20% methanol. The membrane was probed with an anti-FLAG tag antibody (Sigma) followed by a horseradish peroxidase-conjugated goat antimouse IgG antibody (Invitrogen), and then exposed to X-ray film following the addition of appropriate substrates. A similar protocol was used for detection of endogenous S. pombe ValRS1 and ValRS2 (Caspari et al. 2000), except that the antibody used was anti-c-myc antibody (Evan et al. 1985).

Aminoacylation Assay
Aminoacylation reactions were carried out at 25 °C in a buffer containing 10 mM Tris–HCl (pH 7.9), 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 4 mM adenosine-5′-triphosphate, 0.1 mg/ml bovine serum albumin, 0.1 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.

Results

Except for S. pombe, All Yeast Species Possessed a Single ValRS Gene
Previous studies showed that a single VAS1 gene of S. cerevisiae specifies both the mitochondrial and cytoplasmic forms of ValRS. We wondered whether a similar feature of one gene coding for two protein isoforms is conserved in ValRS genes of other yeast species. Pursuant to this objective, 15 different yeast ValRS sequences were retrieved from the EMBL database, and the N-terminal sequences of these proteins were aligned using the ClustalW method (Thompson et al. 1994). Interestingly, except for S. pombe, which contains two distinct nuclear ValRS genes (designated herein as SpVAS1 and SpVAS2), all yeast species studied possessed a single ValRS gene, named VAS1 (fig. 1). Given that both cytoplasmic and mitochondrial aminoacylation activities are required for protein translation in yeast, it is likely that these genes also specify both forms. Consistent with this hypothesis, except for VAS1 of Y. lipolytica, all these genes contain two in-frame ATG initiator candidates near the 5′-end of their respective open reading frames (as exemplified by sequences shown in figs. 1 and 2). Although only one ATG initiator candidate was found in VAS1 of Y. lipolytica, a sequence analysis suggested that an ATT triplet 24 codons upstream of the ATG initiator may serve as an alternative translation initiator codon (fig. 2B).

Also interesting was the discovery that, except for the ValRS sequences of S. pombe, all yeast ValRS sequences deduced from their respective open reading frames possessed an N-terminal mitochondrial targeting signal followed by a lysine-rich Ad (summarized in figs. 1 and 2A). It should be noted that the first and second methionine residues in each of these sequences, respectively, serve as the initiating residues for the mitochondrial and cytoplasmic forms. In addition, the Ads shared a much higher homology than did the mitochondrial targeting signals (fig. 1). In contrast to these dual-functional ValRS genes, SpVAS1 and SpVAS2 appeared to encode a single protein form by having a single initiator codon at the 5′ end of their respective open reading frames. Sequence alignments further revealed that the protein forms deduced from SpVAS1 and SpVAS2 lacked a mitochondrial targeting signal and an Ad, respectively (figs. 1 and 2). This finding suggests that SpVAS1 and SpVAS2, respectively, encode the cytosolic and mitochondrial forms of S. pombe ValRS. Although SpValRS1 closely resembles its yeast homologues (~60% identity in the catalytic core domain), SpValRS2 shares only ~35% identity with others.

The VAS1 Genes of C. albicans and Y. lipolytica Encode Both Cytoplasmic and Mitochondrial Forms of ValRS
To provide experimental evidence for the functional potential of these genes, four yeast ValRS genes, CaVAS1 (VAS1 of C. albicans), YlVAS1, SpVAS1, and SpVAS2, were cloned in appropriate yeast shuttle vectors, and their cross-species complementation activities were assayed in CW1, a VAS1 knockout strain of S. cerevisiae. As shown in figure 2, CaVAS1 and YlVAS1 rescued both the cytoplasmic and mitochondrial defects of the knockout strain; transformants containing either of these two constructs grew well on 5-FOA and YPG plates (fig. 2C–E, “numbers 1 and 4”). Mutations of ATG1 and ATG39 in CaVAS1, respectively, impaired the mitochondrial and cytoplasmic activities (fig. 2C–E, “numbers 2 and 3”), suggesting that ATG1 and ATG39, respectively, serve as translation initiator codons of the mitochondrial and cytoplasmic forms of CaValRS. Similarly, ATT(−24) and ATG1 of YlVAS1, respectively, serve as translation initiator codons of the mitochondrial and cytoplasmic forms of CaValRS (fig. 2C–E, “numbers 5 and 6”). In contrast to the dual-functional phenotype of CaVAS1 and YlVAS1, SpVAS1 rescued only the cytoplasmic defect of CW1 (fig. 2C–E, “number 8”), whereas SpVAS2 rescued neither defects of the knockout strain (fig. 2C–E, “number 7”).
To advance understanding of the functional potential of SpValRS2, an N-terminal segment of this protein (residues 1–31), which was predicted to serve as a mitochondrial targeting signal, was fused to the N-terminus of the cytoplasmic form of S. cerevisiae ValRS (ScValRS), and the complementing activities of the resultant construct was assayed. As shown in figure 3, the cytoplasmic form of ScValRS existed exclusively in the cytoplasm but could...
be targeted to the mitochondria by fusion of the predicted mitochondrial targeting signal of SpValRS2 (compare numbers 2 and 7). This result supports the notion that the N-terminal sequence of SpValRS2 functions as a mitochondrial targeting signal. Similarly, SpValRS1, which was itself restricted to the cytoplasm, could function in the mitochondria by fusion of the mitochondrial targeting signal of the mitochondrial form of ScValRS (N-terminal residues 1–46) (fig. 3A–C, numbers 5 and 6). Unfortunately, deletion of the mitochondrial targeting signal from SpValRS2 did not yield a functional cytoplasmic ValRS (numbers 3 and 4). Western blotting assays further showed that the WT SpValRS2 was poorly expressed in S. cerevisiae, and the N-terminally truncated form of this enzyme was essentially undetectable under the conditions used (fig. 3D, numbers 3 and 4). Thus, it is not clear at the moment whether the negative phenotype of SpValRS2 in the crossspecies complementation assays was due to tRNA specificity or poor protein expression.

Fig. 2. Crossspecies complementation assays for ValRSs from various yeast species. WT and mutant VAS1 constructs were transformed into CW1, and the ability of the constructs to rescue the growth defects of the knockout strain was tested. (A) Comparison of Escherichia coli and yeast ValRSs. (B) 5'-End sequences of ValRS genes from various yeast species. For clarity, the predicted translation initiator codons are shaded. The sequences shown extend from the nearest upstream stop codon to the initiator codon of the cytoplasmic or mitochondrial form. (C) Schematic summary of the VAS1 and VAS2 constructs and their complementation activities. Mit, mitochondrial and Cyt, cytoplasmic. (D) Crossspecies complementation assays for cytoplasmic ValRS activity. (E) Crossspecies complementation assays for mitochondrial ValRS activity. Numbers 1–8 (circled) in C–E denote the constructs shown in C.

Fig. 3. Testing for the mitochondrial targeting signal of Schizosaccharomyces pombe ValRSs. The predicted mitochondrial targeting signal of SpValRS2 was fused at the N-terminus of ScValRS3, and the ability of the fusion construct to rescue the growth defects of CW1 was tested. (A) Schematic summary of the VAS1 and VAS2 constructs and their complementation activities. MTS, mitochondrial targeting signal and Ad. (B) Complementation assays for cytoplasmic ValRS activity. (C) Complementation assays for mitochondrial ValRS activity. (D) Assay of protein expression by Western blotting. Upper panel, ValRS and lower panel, PGK (as the loading control).
mitochondria and nuclei, respectively. A mitochondrial tracker and Hoechst dyes were used to label the localization of SpValRS1 and SpValRS2 by fluorescence microscopy.

Cytosolic and Mitochondrial Forms of ValRS

To determine the relative levels of endogenous SpValRS1 and SpValRS2 proteins in S. pombe, we inserted a short DNA sequence coding for a 13x-myc tag at the 3’ end of these two genes in the yeast chromosomes. Subsequently, total protein extracts were prepared from the recombinant yeast strains and analyzed by Western blotting using an anti-c-myc antibody. As shown in figure 4B, SpValRS1 had an expression level ~40-fold higher than that of SpValRS2. Together with the results of the RT-PCR (fig. 4A), this result indicates that differences in expression levels of these two genes were largely attributable to differences in transcriptional levels. Finally, to confirm the cellular localization of these two proteins in S. pombe, a GFP was independently tagged at the C-terminus of these two proteins. As shown in figure 4C, SpValRS2-GFP existed exclusively in the mitochondria (a–c), whereas SpValRS1-GFP was evenly distributed in the cytosol (d–f). This finding provides strong evidence that SpVAS1 and SpVAS2 encode the cytoplasmic and mitochondrial forms of ValRS, respectively.

SpVAS1 and SpVAS2 Are Both Essential Genes

In the crossspecies complementation assays, SpVAS2 failed to rescue the mitochondrial defect of CW1 (a S. cerevisiae vas1Δ strain) (fig. 2). We wondered whether this gene actually encodes a functional mitochondrial enzyme in S. pombe. To determine the biochemical activity of this enzyme in vitro, recombinant SpValRS2-His6 was purified from a transformant of S. pombe containing a plasmid-borne SpVAS2 gene by Ni-NTA column chromatography to homogeneity, and unfractionated S. pombe tRNA was used as the substrate for the aminoacylation reactions. As controls, recombinant SpValRS1-His6 (serving as a positive control) and SpGlnRS-His6 (serving as a negative control) were also purified and tested. Figure 5 shows that although the aminoacylation activity of SpValRS2-His6 was much lower than that of SpValRS1-His6, both enzymes were truly active in aminoacylation using S. pombe tRNA as the substrate. In contrast, SpGlnRS-His6, which was purified following a similar protocol, appeared completely inactive in coupling [3H]valine to tRNA. Thus, the valylation activity detected in the preparation of SpValRS2-His6 did not result from protein contamination, and SpValRS2-His6 was indeed an active enzyme.

To gain new insights into the biological functions of these two genes, a knockout strain was constructed for each. One of the two alleles of SpVAS1 or SpVAS2 in the yeast chromosomes was first substituted with a URA4 gene in a parental diploid strain of S. pombe, resulting in SpVAS1+/SpVAS1−/SpVAS1+/SpVAS1−. The same sets of primers were subsequently used to amplify the cDNA fragments of SpVAS1 and SpVAS2, which had been reverse-transcribed from a preparation of S. pombe total RNA using oligo-dT as the primer. As shown in figure 4A, the cDNA level of SpVAS1 was ~50-fold higher than that of SpVAS2 ("lower panel").

Next, to determine the relative levels of endogenous SpValRS1 and SpValRS2 proteins in S. pombe, we inserted a short DNA sequence coding for a 13x-myc tag at the 3’ end of these two genes in the yeast chromosomes. Subsequently, total protein extracts were prepared from the recombinant yeast strains and analyzed by Western blotting using an anti-c-myc antibody. As shown in figure 4B, SpValRS1 had an expression level ~40-fold higher than that of SpValRS2. Together with the results of the RT-PCR (fig. 4A), this result indicates that differences in expression levels of these two genes were largely attributable to differences in transcriptional levels. Finally, to confirm the cellular localization of these two proteins in S. pombe, a GFP was independently tagged at the C-terminus of these two proteins. As shown in figure 4C, SpValRS2-GFP existed exclusively in the mitochondria (a–c), whereas SpValRS1-GFP was evenly distributed in the cytosol (d–f). This finding provides strong evidence that SpVAS1 and SpVAS2 encode the cytoplasmic and mitochondrial forms of ValRS, respectively.

**SpVAS1 and SpVAS2, Respectively, Encode the Cytosolic and Mitochondrial Forms of ValRS**

To take a closer look at the endogenous expression and localization of the ValRS isoforms in S. pombe, several different approaches were employed, including reverse transcription-polymerase chain reaction (RT-PCR), Western blotting, and fluorescence microscopy. First, to determine the relative levels of SpVAS1 and SpVAS2 mRNAs in the yeast were determined by RT-PCR using total RNA as the template. As a reference, the same sets of primers were used to amplify the DNA of these two genes using genomic DNA as the template. gDNA, genomic DNA; cDNA, complementary DNA. (B) Analysis of SpVAS1 and SpVAS2 by Western blotting. (C) Analysis of cellular localization of SpVAS1 and SpVAS2 by fluorescence microscopy. A mitochondrial tracker and Hoechst dyes were used to label the mitochondria and nuclei, respectively.

**Fig. 4.** Expression and cellular localization of SpValRS1 and SpValRS2. (A) Analysis of SpVAS1 and SpVAS2 mRNAs by RT-PCR. The relative levels of SpVAS1 and SpVAS2 mRNAs in the yeast were determined by RT-PCR using total RNA as the template. As a reference, the same sets of primers were used to amplify the DNA of these two genes using genomic DNA as the template. gDNA, genomic DNA; cDNA, complementary DNA. (B) Analysis of SpVAS1 and SpVAS2 by Western blotting. (C) Analysis of cellular localization of SpVAS1 and SpVAS2 by fluorescence microscopy. A mitochondrial tracker and Hoechst dyes were used to label the mitochondria and nuclei, respectively.
or \( \text{SpVAS}^\dagger/\text{SpVAS}^\dagger/\text{SpVAS}^\dagger \). The heterozygotes were then allowed to sporulate, and the growth phenotypes of the resulting spores were tested on YE35 medium (0.5% yeast extract, 2% glucose, 225 \( \mu \text{g/ml} \) each of adenine, leucine, and uracil) after tetrad dissection. As shown in figure 6A, a single DNA band of \( \sim 3.4 \text{ kb} \) was amplified from the parental diploid strain using primers specifically complementary to the 5' and 3' untranslated regions of \( \text{SpVAS}^2 \) or \( \text{SpVAS}^1 \) (“numbers 1 and 3”), whereas an extra DNA band of \( \sim 1.5 \text{ kb} \) was amplified from the heterozygotes (“numbers 2 and 4”). The \( \sim 3.4\)-kb DNA band corresponds to the size of \( \text{SpVAS}^1 \) or \( \text{SpVAS}^2 \), whereas the \( \sim 1.5\)-kb DNA band corresponds to the size of \( \text{URA}4 \). This result clearly verifies that one of the two alleles of \( \text{SpVAS}^1 \) or \( \text{SpVAS}^2 \) was replaced by \( \text{URA}4 \) in the respective heterozygotes. After sporulation, tetrads were dissected under a dissection microscope and then spotted on a YE35 agar plate for growth. Figure 6B shows that only two of the four spores derived from each tetrad grew on the plate, irrespective of the gene that was deleted, suggesting that \( \text{SpVAS}^1 \) and \( \text{SpVAS}^2 \) are both essential for the yeast. Despite that, it was interesting to note that the haploid strain \( \text{SpVAS}^2^- \) (\( \text{SpVAS}^2+:\text{URA}4^+ \)) could form microcolonies before its growth was completely stopped (fig. 6B, left panel, tetrad 1), whereas the haploid strain \( \text{SpVAS}^1^- \) (\( \text{SpVAS}^1+:\text{URA}4^- \)) failed to germinate at all.

**SpVAS2 Appears to Be Ancestral to SpVAS1**

As \( \text{SpValRS}^2 \) (the mitochondrial form) has significantly diverged from \( \text{SpValRS}^1 \) (the cytoplasmic form) and ValRSs from other yeast species, possible historical relationships between \( \text{SpValRS}^2 \) and its yeast homologues were analyzed using the Neighbor-Joining method. To expand the scope of this phylogeny, representative ValRS sequences from all three of the major branches of life (bacteria, archaea, and eukarya) were also subjected to analysis. Bias in the alignments of these sequences was minimized by reducing them to their core active site. This portion comprises only \( \sim 65\% \) of the sequence of \( S. \text{ pombe} \) ValRS2. As shown in figure 7, \( \text{SpValRS}^2 \) and \( \text{SpValRS}^1 \) were clustered within a monophyletic branch with ValRS sequences from other eukaryotes, including \( S. \text{ cerevisiae}, C. \text{ albicans}, Y. \text{ lipolytica}, A. \text{ thaliana}, Drosophila melanogaster \) (the cytoplasmic and mitochondrial isoforms), and \( \text{Homo sapiens} \) (the cytoplasmic and mitochondrial isoforms), with \( \text{SpValRS}^2 \) being more distantly related to others (fig. 7). Note that as with the case in \( S. \text{ cerevisiae} \), a single \( \text{VAS}^1 \) gene encodes both forms of ValRS in \( A. \text{ thaliana} \) (Souciet et al. 1999). In contrast to the remote relatedness of \( \text{SpValRS}^2 \), \( \text{SpValRS}^1 \) appeared to have evolutionarily diverged from other yeast ValRSs only recently. Most interestingly, except for \( \text{SpValRS}^2 \) and \( \text{DmValRS}^2 \) (presumably the mitochondrial form of \( D. \text{ melanogaster} \) ValRS), all the eukaryotic ValRS sequences shown here contained an N-terminal Ad, which is believed to have been added to the enzymes later during evolution. These findings altogether suggest that \( \text{SpValRS}^2 \) is ancestral to \( \text{SpValRS}^1 \), and may represent an ancient form of eukaryotic ValRS. As for ValRS sequences from archaea and bacteria, they appeared to cluster independently into two paraphyletic branches, with the bacterial branch being more closely related to the eukaryotic branch.
Discussion

In the work reported here, we studied the function and phylogeny of the ValRS genes from 14 yeast species. Our results showed that, except for *S. pombe*, all the yeast species studied possessed a single ValRS gene encoding both mitochondrial and cytoplasmic forms (figs. 1 and 2). Thus, it appears that a single gene with dual functions is a common theme for the majority of yeast ValRS genes. In contrast, *S. pombe* possesses two distinct nuclear genes that encode the mitochondrial and cytoplasmic forms of ValRS (figs. 4 and 5). Despite the fact that *S. pombe* ValRS2 is active in vitro and in vivo (figs. 4–6), a sequence comparison indicated that it lacks the characteristic N-terminal Ad that is conserved in SpValRS1 and other yeast homologues (figs. 1 and 7). Paradoxically, like SpValRS1, SpValRS2 is also essential for survival of the yeast even under conditions suitable for fermentation (fig. 6). A similar scenario was recently reported for Bot1p, a nuclear-encoded mitochondrial protein involved in mitochondrial protein synthesis in *S. pombe* (Wiley et al. 2008). These findings provide further support for the notion that *S. pombe* is a petite-negative yeast and cannot survive the loss of mitochondrial DNA and mitochondrial protein synthesis (Schafer 2003).

A general feature regarding the Ads of yeast tRNA synthetases is their intrinsic capability to nonspecifically bind to RNA (Mirande 1991). Such activity is thought to enhance the overall efficiency of aminoacylation by recruiting tRNA to the catalytic core. The binding affinity of a tRNA synthetase toward its cognate tRNAs is generally characterized by dissociation constants of the order of 0.1–1 μM under physiological conditions (Schimmel and Soll 1979). This relatively low affinity ensures that the synthetases (or tRNAs) turn over rapidly during aminoacylation and translation. Unlike the Ad of yeast GlnRS, for which large deletions in the Ad had little effect on the in vitro aminoacylation activity (Ludmerer et al. 1993) or in vivo complementation activity (Ludmerer and Schimmel 1987), the Ad of yeast ValRS played a much more important role in the biochemical activity of the enzyme. Deletion of the Ad from the yeast enzyme severely impaired its tRNA-binding, aminoacylation, and complementation activities (Chang et al. 2008). Perhaps for this reason, ValRS from almost all yeast species studied so far possesses a similar N-terminal Ad (figs. 1 and 2). Moreover, our recent study on yeast ValRS suggested that an appended nonspecific tRNA-binding domain is more important and thus indispensable for the cytoplasmic enzyme than for its mitochondrial counterpart (Chiu et al. 2009). In this connection, the finding that SpValRS2, a ValRS enzyme without an Ad, can function in the mitochondria of *S. pombe* was not totally unexpected (figs. 4 and 6). Conversely, SpValRS1, a ValRS enzyme with an Ad, when forced into mitochondria, can function as a mitochondrial enzyme (fig. 3). This finding suggests that an Ad may provide a selective advantage for the enzyme and pave the way for the evolution of a dual-functional gene (Chen et al. 2009). Although the Ads of yeast GluRS and MetRS are also rich in positively charged residues and important for aminoacylation, they do not function as tRNA-binding domains.

**Fig. 7.** Phylogenetic analysis of the relationship of SpValRS1, SpValRS2, and other ValRSs. The sequences comprising of the core active sites of ValRSs were aligned with ClustalW (22) and analyzed using the Neighbor-Joining method. The numbers at the nodes denote bootstrapping frequencies calculated from 1,000 trees. (A) fulgidus, *Archaeoglobus fulgidus* (NP_071049); (A) thaliana, *Arabidopsis thaliana* (NP_172913); (B) subtilis, *Bacillus subtilis* (NP_390687); (C) crescentus, *Caulobacter crescentus* (NP_420133); D. melanogaster, *Drosophila melanogaster* (DmValRS1, NP_524838; DmValRS2, NP_648268); E. coli, *Escherichia coli* (NP_418679); H. sapiens, *Homo sapiens* (HsValRS1, NP_006286; HsValRS2, NP_065175); M. jannaschii, *Methanocaldococcus jannaschii* (NP_248001); M. thermotrophicus, *Methanothermobacter thermautotrophicus* (NP_275909).
Instead, these Ads specifically interact with a tRNA-binding cofactor, Arc1p, which, in turn, recruits tRNA to the associated enzymes for aminoacylation (Simos et al. 1998). A functionally similar tRNA-recruiting domain was identified in an auxiliary protein associated with the mammalian multisynthetase complex (Shalak et al. 2001).

A ValRS phylogeny rooted by isoleucyl-tRNA synthetases showed that eukaryotic ValRS sequences are clustered into a monophyletic branch that displays high affinity to gram-negative bacteria (Brown and Doolittle 1995). On the basis of this finding and others, it was proposed that the ValRS gene in mitochondrion-containing eukaryotes was horizontally transferred to the nucleus from an ancestral mitochondrion, and all contemporary nuclear-encoded ValRS proteins of eukaryotes are regarded as being of mitochondrial origin (Brown and Doolittle 1997).

Surprisingly, ValRS sequences from Giardia lamblia and Trichomonas vaginalis, both of which are amitochondriate protists, are also of mitochondrial origin (Hashimoto et al. 1998), suggesting that evolution of ValRS in all eukaryotes may have followed a similar endosymbiotic pathway. In this sense, the discovery that S. pombe possesses two distinct nuclear ValRS genes is of particular interest (figs. 1 and 2). Despite the fact that SpValRS1 and SpValRS2 have significantly diverged from each other (~35% identity), both proteins possess the signature sequence of ~37 residues downstream of the KMSKS motif (one of the two conserved motifs characteristic of all class I aaRSs) that distinguishes the eukaryotic and γ-proteobacterial ValRS sequences from all others (Hashimoto et al. 1998) and are clustered into a monophyletic branch with other eukaryotic ValRS sequences (fig. 7). These features, together with the fact that SpValRS2 lacks the characteristic Ad conserved in all of its yeast homologues, strongly support the hypothesis that the mitochondrial form of S. pombe ValRS represents a primitive form of eukaryotic ValRS of mitochondrial origin. And so, the gene encoding the cysteine-class ValRS1 may have evolved from a gene duplication event, followed by acquisition of an N-terminal Ad.

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References


