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The yeast protein Arc1p binds to tRNA and functions as a cofactor for the methionyl- and glutamyl-tRNA synthetases

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Arc1p was found in a screen for components that interact genetically with Los1p, a nuclear pore-associated yeast protein involved in tRNA biogenesis. Arc1p is associated with two proteins which were identified as methionyl-tRNA and glutamyl-tRNA synthetase (MetRS and GluRS) by a new mass spectrometry method. *ARC1* gene disruption leads to slow growth and reduced MetRS activity, and synthetically lethal arc1⁻ mutants are complemented by the genes for MetRS and GluRS. Recombinant Arc1p binds *in vitro* to purified monomeric yeast MetRS, but not to an N-terminal truncated form, and strongly increases its apparent affinity for $tRNA^{Met}$. Furthermore, Arc1p, which is allelic to the quadruplex nucleic acid binding protein G4p1, exhibits specific binding to tRNA as determined by gel retardation and UV-cross-linking. Arc1p is, therefore, a yeast protein with dual specificity: it associates with tRNA and aminoacyl-tRNA synthetases. This functional interaction may be required for efficient aminoacylation *in vivo*.

Keywords: aminoacyl-tRNA synthetase/ARC1/Los1p/nuclear pore complex/tRNA aminoacylation

Introduction

The biogenesis of tRNA is a complex process taking place in different cellular compartments and involving many consecutive steps before the mature tRNA is delivered to the ribosomes; this includes transcription, trimming, modification, splicing, transport from the nucleus to the cytoplasm and aminoacylation (for reviews, see Hopper and Martin, 1992; Meinnel *et al.*, 1995). So far, little is known on how these various steps are controlled and whether they depend on each other. On the other hand it is clear that the structural integrity of the tRNA molecule is a prerequisite for efficient nuclear export (Tobian *et al.*, 1985), and unspliced or partially processed pre-tRNAs cannot exit from the nucleus (Haselbeck and Greer, 1993). For intron-containing pre-tRNAs, it has been suggested that the tRNA-splicing reaction may be coupled to the translocation step, since the key enzymes of splicing, the tRNA endonuclease and tRNA ligase, were found to be localized at the inner side of the nuclear membrane, in close proximity to the nuclear pore complex (Westaway and Abelson, 1995). It has also been shown recently that in yeast, several nucleoporin mutants are defective in pre-tRNA splicing as well as in biogenesis of active suppressor tRNA (Sharma *et al.*, 1996; Simos *et al.*, 1996), suggesting that pre-tRNA splicing and nuclear export of tRNA require normal nuclear pores. Furthermore, modification of tRNA also appears to be functionally coupled to the nuclear export process (Simos *et al.*, 1996). Processing of tRNA in the nucleus is in most cases mediated by proteins that recognize the common tertiary structure of tRNAs. However, once in the cytoplasm, the mature forms of tRNAs have to encounter and interact with their cognate aminoacyl-tRNA synthetases, in order to be charged with the corresponding amino acids (Schimmel, 1987; Meinnel *et al.*, 1995). Although they carry out the same reaction, aminoacyl-tRNA synthetases differ significantly in their primary sequence as well as in the size and the number of their subunits. The quaternary structure of individual aminoacyl-tRNA synthetases is conserved between prokaryotes and eukaryotes with only a few exceptions such as in the case of methionyl-tRNA synthetase (MetRS). The *Escherichia coli* MetRS is a dimer made up of two identical subunits, but removal of 120 amino acids from the C-terminus by mild proteolysis or genetic engineering generates a monomeric active enzyme fragment, the crystal structure of which has been solved (reviewed in Meinnel *et al.*, 1990). In contrast, purified yeast MetRS behaves as a monomer and comparison of the primary sequences of yeast and *E.coli* MetRS indicates that the yeast enzyme possesses a N-terminal extension of 200 amino acids, which is largely dispensable for activity (Walter *et al.*, 1989), but lacks the C-terminal domain, present on its prokaryotic counterpart (Fasiolo *et al.*, 1985). N-terminal extensions also occur in other aminoacyl-tRNA synthetases from yeast and higher eukaryotes (Kisselev and Wolfson, 1994). However, these N-terminal domains appear in several cases not to be essential for the enzymatic function (Mirande, 1991). In higher eukaryotes, several aminoacyl-tRNA synthetases, including MetRS, are associated together to form a supramolecular multi-enzyme complex (Kerjan *et al.*, 1994). The function of this complex, which contains besides nine synthetases, three non-enzymic polypeptides of 43, 38 and 18 kDa, remains largely unknown (Mirande, 1991). Despite some controversy (Harris and Kolanko, 1995), it has been largely thought that a supramolecular assembly of aminoacyl-tRNA synthetases does not exist in yeast (Mirande, 1991). Apart from the components of the translation machinery, there are only a few proteins which are known to be able to associate specifically with tRNA. One of them is glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

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which binds to tRNA (Singh and Green, 1993) as well as to AU-rich RNA (Nagy and Rigby, 1995); another is the nuclear protein zuotin, which is able to bind to Z-DNA (Zhang *et al.*, 1992) or tRNA (Wilhelm *et al.*, 1994). However, no evidence is available for the functional role of these proteins.

We have recently shown that the yeast protein Los1p, which was initially identified as a component involved in tRNA biogenesis (Shen *et al.*, 1993), interacts genetically with nuclear pore proteins and is itself localized at the nuclear pores (Simos *et al.*, 1996). To gain further insight into the function of Los1p we searched for its interacting proteins in yeast using the method of synthetic lethality. We report here, that Los1p interacts genetically with the cytoplasmic protein Arc1p which forms a complex with two aminoacyl-tRNA synthetases, methionyl-tRNA synthetase (MetRS) and glutamyl-tRNA synthetase (GluRS). Furthermore, we show that Arc1p is itself a tRNA-binding protein which upon association with MetRS increases its affinity for its cognate tRNA.

Results

Identification of Arc1p as a component which interacts genetically with Los1p, a nuclear pore-associated protein involved in tRNA biogenesis

We have recently shown that Los1p is functionally linked to several components including the nuclear pore protein Nsp1p and an intranuclear tRNA pseudouridine synthase (Simos *et al.*, 1996). To further elucidate the role of Los1p at the nuclear pore complex, we sought to identify more of its interacting components. Therefore, a synthetic lethal (sl) screen was performed with a *los1* disruption allele (*LOS1* is not essential in yeast), employing a red/white colony sectoring assay as described earlier (Wimmer *et al.*, 1992). Several sl mutants were isolated in which a mutation in a single gene gave rise to a lethal phenotype when *LOS1* was disrupted, but were viable when *LOS1* was re-expressed. Synthetic lethality is interpreted as genetic evidence of physical interaction or functional overlap of the gene products. Mutations in two complementation groups were obtained, one of which has been recently described (Simos *et al.*, 1996). The second group of mutants (one representative of this group was called sl29) was complemented by a novel gene which was named *ARC1* (Figure 1A) for aminoacyl-tRNA synthetase cofactor 1 (see below). During the course of this work, the same gene product was identified as the quadruplex nucleic acid binding protein G4p1 (Frantz and Gilbert, 1995) (see also Discussion).

An independent proof that *LOS1* and *ARC1* functionally overlap is to show that a synthetically lethal phenotype can be generated by combining defined mutant alleles of *los1* and *arc1*. Gene disruption of *ARC1* (see Materials and methods) showed that it is not essential, although the $arc1^-$ mutant grows slowly as compared with $ARC1^+$ cells over a broad range of temperature, with a distinct cold-sensitive growth phenotype, particularly at lower temperatures (between 23°C and 15°C) (Figure 1C). A haploid yeast strain in which the $los1^-$ and $arc1^-$ gene disruptions are combined, is not viable even at 30°C. This synthetic lethal phenotype can be complemented when the double mutant is transformed with either of the wild-type genes, *LOS1* and *ARC1*, respectively (Figure 1B). Thus, Los1p and Arc1p functionally overlap in the living cell.

Sequencing of *ARC1* showed that it encodes a basic protein (pI = 8.99) of 376 amino acids with a predicted molecular mass of 42 kDa (Figure 2A). Three domains can be discerned within Arc1p. An N-terminal domain (residues 1–131) with little or no homology to known proteins, a middle domain (residues 132–200) rich in lysine and alanine residues which shows sequence similarity (34% identity) to H1 histones, and a C-terminal domain (residues 201–376) which exhibits significant homology to several other proteins in the data libraries with the highest one (54% identity) to the human protein EMAP II (endothelial-monocyte-activating polypeptide II) (Kao *et al.*, 1994). Strikingly, the first half of the C-domain of Arc1 is also homologous (34% identity) to the C-terminus of prokaryotic methionyl-tRNA synthetase (Figure 2C) and, to a lesser extent (29% identity), to the N-terminus of the β chain of prokaryotic phenylalanyl-tRNA synthetase (Figure 2B). According to this multiple sequence alignment, a 100 amino acid sequence within the C-terminal domain of Arc1p (from residue 210 to 300) appears to constitute a novel sequence motif or module which is conserved throughout evolution and found in organisms as diverse as bacteria and humans (Figure 2B).

Arc1p physically and functionally interacts with the methionyl- and glutamyl-tRNA synthetases

The homology between Arc1p and distinct domains in prokaryotic aminoacyl-tRNA synthetases, and the fact that Arc1p interacts genetically with Los1p, was suggestive of a role of Arc1p in the tRNA biogenesis pathway. To find out whether Arc1p associates physically with other proteins in the cell, Arc1p was affinity-purified as an epitope-tagged fusion protein containing the IgG-binding domain derived from *Staphylococcus aureus* protein A (see Materials and methods). ProtA–Arc1p was functional since it could complement the synthetic lethal phenotype of sl29 and the cold-sensitive phenotype of the $arc1^-$ strain (data not shown). When the bound ProtA–Arc1p was finally eluted from the IgG–Sepharose column, the eluate contained, besides ProtA–Arc1p, an additional doublet band migrating at ~85 kDa on SDS–PAGE (Figure 3A, lane 4). These associated proteins could not be removed from ProtA–Arc1p prebound to the IgG–Sepharose column by washing with 1 M NaCl (data not shown), but were eluted by increasing concentrations of $MgCl_2$. Whereas the lower band of the doublet dissociated from the column predominantly at 1 M $MgCl_2$, the upper band detached mainly at 4 M $MgCl_2$, together with the ProtA–Arc1p fusion protein (Figure 3B). To identify these ProtA–Arc1p-associated polypeptides, peptide sequence data were obtained from the corresponding protein bands by a new mass spectrometric method (Wilm *et al.*, 1996). Both bands contained ~1–3 pmol of protein as judged by intensity of staining. They were excised together and nine peptides were sequenced in the unseparated peptide mixture by tandem mass spectrometry in a single experiment (Figure 3C). In a database search using peptide sequence tags (Mann and Wilm, 1994), four peptides covering 49 amino acids uniquely mapped to yeast MetRS

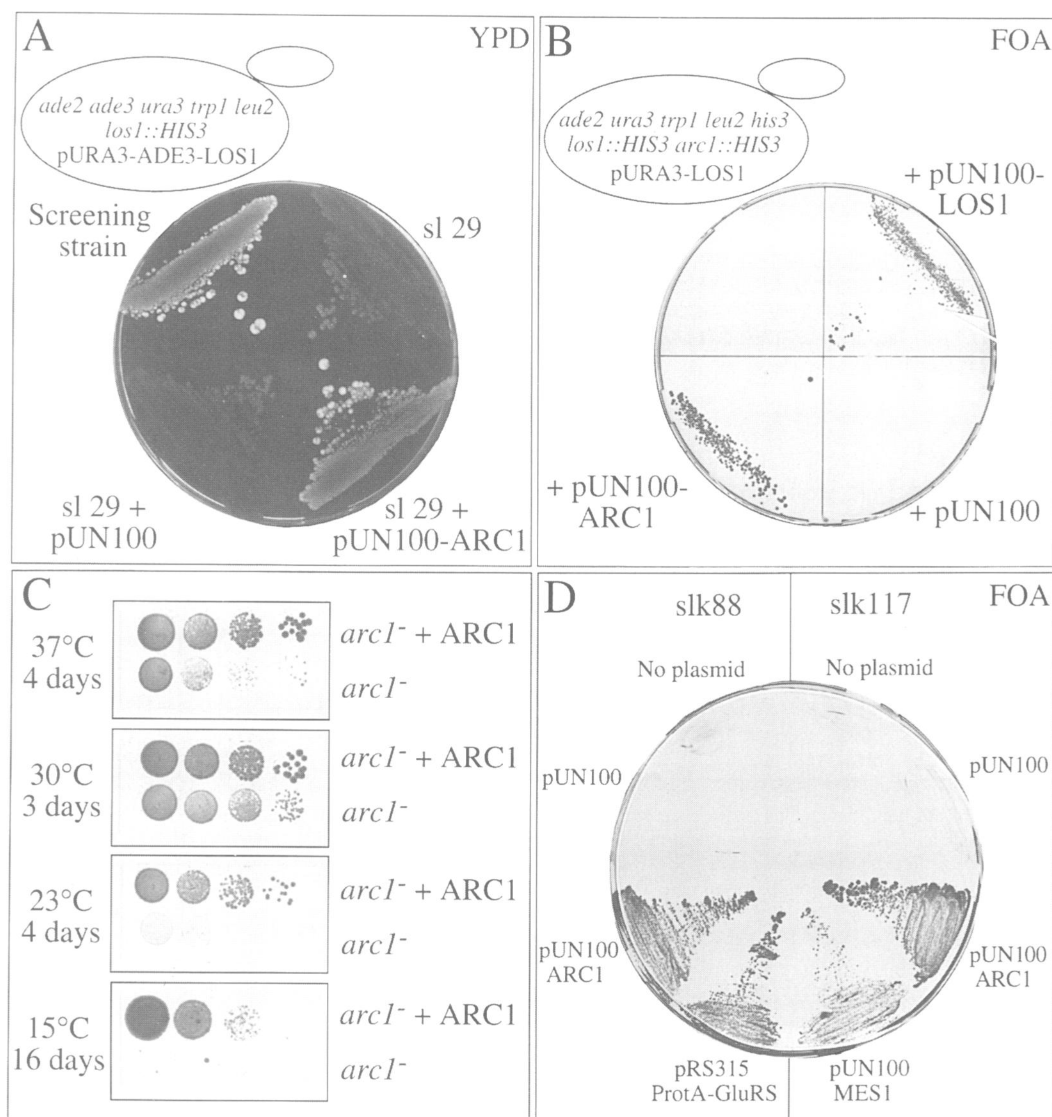

Fig. 1. Arc1p genetically interacts with Los1p and two aminoacyl-tRNA synthetases. (**A**) Red/white colony sectoring on YPD plates is restored in the los1 synthetic lethal strain, sl29, by transformation with the *ARC1* gene (pUN100–ARC1), but not with an empty plasmid (pUN100). (**B**) The double mutant los1⁻ arc1⁻ carrying a pURA3–LOS1 plasmid is unable to grow in the presence of 5-FOA (cannot lose the pURA3–LOS1 plasmid) unless transformed by the wild-type *LOS1* or *ARC1* genes. (**C**) Disruption of *ARC1* leads to slow growth at all temperatures and a cold-sensitive phenotype. (**D**) Two arc1 synthetic lethal mutants, slk117 and slk88, can be complemented by transformation with the genes for MetRS (*MES1*) or ProtA–GluRS, respectively. Complementation is shown by the ability to lose the *ARC1* carrying plasmid and grow in presence of 5-FOA.

and eight peptides covering 104 amino acids uniquely mapped to cytoplasmic GluRS. We could also confirm the identity of the faster-migrating band of the doublet as MetRS by immunoblotting using antibodies raised against purified yeast MetRS (Figure 3B). Similar results were also obtained when authentic Arc1p was immunoprecipitated using affinity-purified antibodies raised against recombinant His-tagged Arc1p (data not shown). In order to confirm the association of GluRS with Arc1p, the DNA coding for yeast GluRS was obtained by PCR and a ProtA-tagged form of GluRS was produced. When ProtA–GluRS was affinity-purified from an ARC1^{+} strain, Arc1p, as well as MetRS, co-purified with ProtA–GluRS (Figure 4, lanes 1 and 3) showing in an independent way that Arc1p forms a trimeric complex with two different aminoacyl-tRNA synthetases in yeast. Moreover, when ProtA–GluRS was purified from an arc1^{-} strain, neither Arc1p nor MetRS co-purified, suggesting that GluRS and MetRS do not directly interact, but require Arc1p for complex formation (Figure 4, lanes 2 and 4). As we could not detect any further proteins in association with either ProtA–Arc1p or ProtA–GluRS we conclude that Arc1p exhibits binding specificity and forms a biochemically stable complex only with MetRS and GluRS.

Whereas the biochemical data have clearly demonstrated that Arc1p physically interacts with two different aminoacyl-tRNA synthetases in yeast, the functional importance of this interaction for a living cell was not clear. To identify the components that interact functionally with Arc1p *in vivo*, we performed a synthetic lethal (sl) screen with the *arc1:HIS3* disruption allele, as described above. Sl mutants were obtained which could be grouped into three complementation groups. The first group was complemented by the *LOS1* gene, confirming that *ARC1* and *LOS1* interact genetically in a highly specific manner. Interestingly, groups II and III were complemented by the structural genes

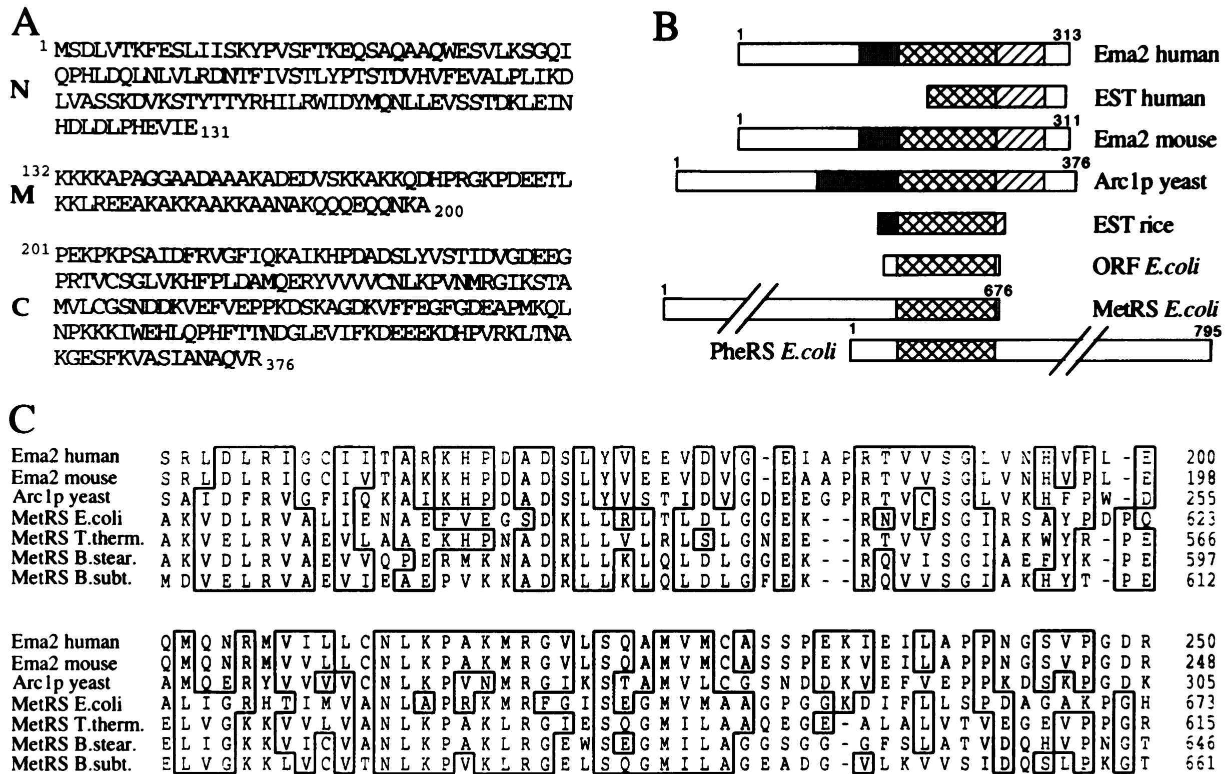

Fig. 2. Arc1p is homologous to both mammalian and prokaryotic proteins. (**A**) The amino acid sequence of Arc1p. The N-terminal, middle and C-terminal domains are indicated. (**B**) Schematic alignment of Arc1p with human and mouse EMAPII, a human (H10555; 45% identity) and a rice (D23020; 58% identity) EST, an uncharacterized *E.coli* ORF (P42589; 38% identity), methionyl-tRNA synthetase (MetRS; P00959) and phenylalanyl-tRNA synthetase (PheRS; P07395) from *E.coli*. The degree of homology of Arc1p to EMAPII, MetRS and PheRS is mentioned in the text. Grey boxes indicate the histone H1 homologous regions, hatched boxes part of the Arc1p C-terminal domain conserved in eukaryotes and cross-hatched boxes the region conserved in both eukaryotes and prokaryotes. (**C**) A 100 amino acid region of the C-terminal domain of Arc1p is homologous to the C-terminal domain of the mouse (U10118) and human (U10117) EMAPII proteins, and to the C-terminal domain of methionyl-tRNA synthetases (MetRS) from *E.coli* (P00959), *T. thermophilus* (P23395), *B. stearothermophilus* (P23920) and *B. subtilis* (P37465). Alignment of the sequences is done by the GCG program 'pileup/prettyplot'.

encoding yeast MetRS and GluRS, respectively (Figure 1D). This shows that mutations in either MetRS or GluRS, which *per se* are tolerated by the cell, cause synthetic lethality when the *ARC1* gene was disrupted. Therefore, the physical interaction between Arc1p, MetRS and GluRS has also important functional relevance.

Recombinant Arc1p binds to MetRS in vitro and stimulates the catalytic efficiency of aminoacylation by lowering the K_m for $tRNA^{Met}$

Using crude cell extracts from $ARC1^+$ and $arc1^-$ strains as a source to measure MetRS activity, we noticed that at saturating tRNA concentrations the $arc1^-$ strain displayed at least a 2.5-fold reduction in the $tRNA^{Met}$ aminoacylation rate and a K_m value for $tRNA^{Met}$ that is more than ten times increased as compared with an $ARC1^+$ strain (Table I). This suggested that the association of Arc1p with MetRS stimulated its activity by affecting the kinetic properties of MetRS for $tRNA^{Met}$. To analyse this relationship further, we wanted to reconstitute *in vitro* the complex formation between Arc1p and MetRS and the stimulation of MetRS activity by using recombinant $(His)_6$-tagged Arc1p and monomeric full-length MetRS ($MetRS_N$), purified from an overproducing yeast strain (Despons *et al.*, 1992). When these two proteins were incubated together, they readily formed a heterodimeric complex since immunoprecipitation of Arc1p with affinity-purified anti-Arc1p antibodies from the incubation mixture led to the specific recovery of MetRS in the immune pellet (Figure 5A, upper panel). In contrast, when a truncated form of MetRS which lacks the N-terminal domain (MetRSΔ6-185), but retains the activity and the specificity of the monomeric full-length MetRS (Walter *et al.*, 1989) was used in the reconstitution assay, no binding to Arc1p could be detected (Figure 5A, lower panel). These data suggest that the first 185 amino acids of native yeast MetRS, which are missing from the prokaryotic enzyme, mediate the physical interaction with Arc1p, probably by containing an Arc1p binding site.

In order to investigate the effect of Arc1p binding to MetRS on MetRS activity, recombinant Arc1p was preincubated with either of the two MetRS forms and the kinetics of aminoacylation of $tRNA^{Met}$ were analysed. The methionylation of tRNA was significantly stimulated when Arc1p was mixed with the intact MetRS (Figure 5B), whereas the activity of N-terminally truncated MetRS was not affected by the presence of Arc1p (data not shown). Compared with $MetRS_N$ alone or MetRSΔ6-185, the k_{cat}/K_m value of $MetRS_N$ (a measure of its catalytic efficiency) increased 500-fold when Arc1p is included into the aminoacylation assay. This increase is mainly manifested at the level of K_m for tRNA which is dramatically reduced (Table I). Therefore, Arc1p by interacting with intact $MetRS_N$ increases its apparent affinity for tRNA.

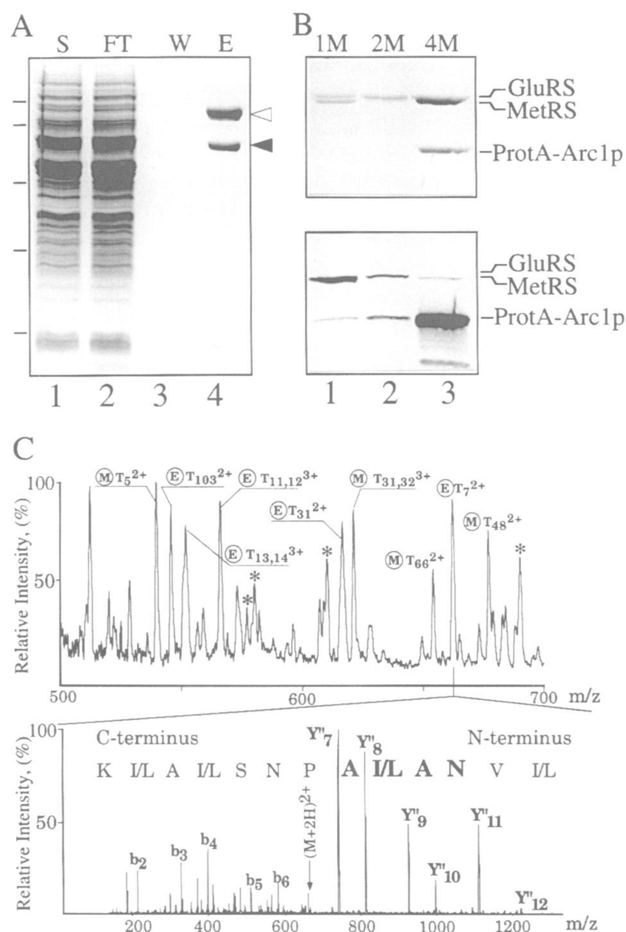

Fig. 3. Identification of the Arc1p associated proteins. (**A**) Affinity-purification of ProtA–Arc1p by IgG–Sepharose chromatography. Fractions of the soluble total cell extract (S), the column flow-through (FT), the pH 5 wash (W) and the pH 3.4 eluate (E) were analysed by SDS–PAGE and Coomassie staining. The black arrowhead indicates the ProtA–Arc1p and the white arrowhead the co-purifying protein doublet. Bars on the left correspond to molecular weight markers (97, 66, 45, 31 and 21 kDa). (**B**) Elution of the bound material from the IgG–Sepharose column with increasing concentrations of $MgCl_2$ (1, 2 and 4 M). Upper panel: Coomassie staining; lower panel: Western blot with anti-MetRS polyclonal antibodies (which also bind to the Protein A moiety of ProtA–Arc1p). (**C**) Part of mass spectrum of the peptide mixture extracted after in gel digestion of the doublet band shown in (B) (lane 1). All labelled peaks were sequenced by tandem mass spectrometry. Peaks annotated by circled M and E were found to correspond to peptides from methionyl-tRNA synthetase and glutamyl-tRNA synthetase, respectively, through database searches. The numbers designate the position of the tryptic peptide in the deduced amino acid sequence and the charge state of the peptide. Peaks labelled with the asterisks are trypsin autolysis products. The approach used for peptide identification is illustrated in the lower panel. The doubly charged ion with mass-to-charge ratio 662.4 was fragmented in the mass spectrometer and tandem mass spectra were obtained. A short sequence stretch was retrieved from the mass differences between adjacent, C-terminal containing (Y″) fragment ions [NA(I/L)A]. Together with the peptides molecular weight they were assembled into peptide sequence tag by the program PeptideSearch version 2.6. A database search by this tag and tags assembled from the other peaks marked with (E) uniquely retrieved peptides originating from glutamyl-tRNA synthetase (accession number P46655).

Arc1p binds specifically to tRNA

Since Arc1p increased the affinity of MetRS for tRNA (as seen in the *in vitro* reconstitution experiment), we examined the possibility that Arc1p can by itself bind to tRNA. Recombinant Arc1p, which was further purified by ion-exchange chromatography, was incubated with radiolabelled *in vitro* transcribed human initiator $tRNA^{Met}$ and the protein–tRNA complexes formed were analysed in a gel retardation assay. This revealed that Arc1p produced a shift of radiolabelled tRNA and therefore is able to bind to tRNA (Figure 6A). Furthermore, when the amount of protein present in the assay is increased, protein–tRNA complexes of slower mobility were formed, suggesting the formation of higher-order complexes. To examine the specificity of the Arc1p–$tRNA^{Met}$ interaction, we performed a series of competition experiments, in the presence of near physiological salt concentration (150 mM NaCl), using *in vitro* transcribed and gel-purified RNA molecules (Figure 6B and C). Binding of Arc1p to yeast ^{32}P-labelled $tRNA^{Met}$ was competed by an excess of unlabelled $tRNA^{Met}$ in a concentration-dependent fashion. Similar degree of inhibition was also observed when other unlabelled yeast tRNAs such as $tRNA^{Phe}$, minor $tRNA^{Ile}$ or intron-containing pre-$tRNA^{Ile}$ and pre-$tRNA^{Phe}$, were used as competitors instead of $tRNA^{Met}$ (Figure 6B and C and data not shown). In contrast, an unstructured RNA

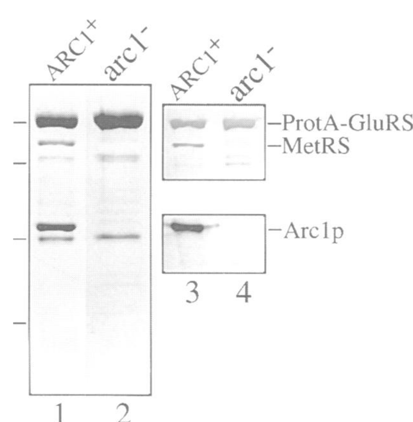

Fig. 4. Arc1p forms a complex with MetRS and GluRS. ProtA–GluRS was affinity-purified by IgG–Sepharose chromatography from a wild-type (lanes 1 and 3) or an arc1⁻ strain (lanes 2 and 4) and fractions of the eluates were analysed by SDS–PAGE and Coomassie staining (lanes 1 and 2) or Western blotting with anti-MetRS (lanes 3 and 4, upper panel) or anti-Arc1p (lanes 3 and 4, lower panel) antibodies. The anti-MetRS antibodies react also with the Protein A moiety of the ProtA–GluRS fusion protein. Note the presence of MetRS and Arc1p in the eluate from the wild-type strain and their absence from the eluate of the *arc1* disrupted strain. Weakly stained bands below ProtA–GluRS in lanes 2 and 4 correspond to degradation products. Bars on the left correspond to molecular weight markers (97, 66, 45 and 31 kDa).

Table I. Determination of catalytic constants of MetRS for $tRNA^{Met}$ in the presence and absence of Arc1p

	Crude extracts from strains		Purified MetRS	
	ARC1⁻	arc1⁻	+Arc1p	–Arc1p
K_m (μM)	<1	8.3	≤0.1	6.6
k_{cat} (min⁻¹)	40[a]	16[a]	6.3	1.3

The source of MetRS was either crude protein extract from wild-type (ARC1⁻) or *ARC1* disrupted (arc1⁻) strain or purified MetRS in the presence and absence of recombinant Arc1p. Initial rates of $tRNA^{Met}$ aminoacylation were determined as described in Materials and methods.

[a]These values correspond to specific activity in pmol/min/mg of protein present in the crude extracts.

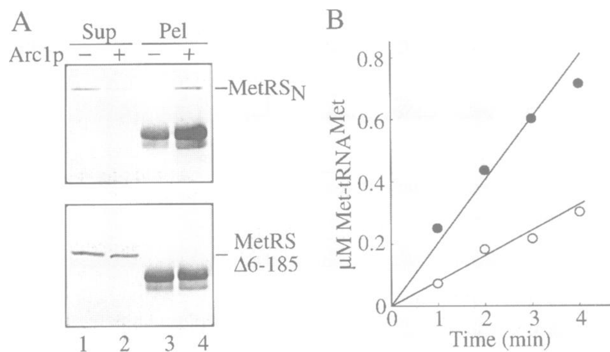

Fig. 5. Recombinant Arc1p binds *in vitro* to MetRS and stimulates its activity. (**A**) Full-length $MetRS_N$ (upper panel) or truncated MetRSΔ6-185 (lower panel) were incubated alone or with recombinant Arc1p, and the formation of a complex was monitored by adding anti-Arc1p antibodies and Protein A–Sepharose beads and probing the material bound on the pelleted beads (Pel) or the supernatant (Sup) with anti-MetRS antibodies. $MetRS_N$ can be readily detected in the Arc1p immunoprecipitate while it is absent from the control sample. In contrast, MetRSΔ6-185 cannot be detected in the immune pellet. The heavily stained bands correspond to the IgG heavy chains. (**B**) Time-course of the activity of purified monomeric $MetRS_N$ pre-incubated in the absence (○) or presence (●) of recombinant Arc1p before dilution into the aminoacylation assay.

species of comparable length (KS RNA; an *in vitro* transcript of the Bluescript KS polylinker region) could not compete for the binding of Arc1p to $tRNA^{Met}$ (Figure 6B and C). To investigate the specificity of Arc1p for structured RNAs we used as competitors the 120 nucleotide 5S rRNA and the 165 nucleotide U1ΔSm snRNA (Jarmolowski *et al.*, 1994). No inhibition of the Arc1p-$tRNA^{Met}$ binding was observed by the U1ΔSm snRNA and the 5 S rRNA inhibited only partially (Figure 6B and C). These results showed that Arc1p interacts specifically with tRNA. To examine further this property, we used RNA minihelices corresponding to the two branches of the L-form of yeast $tRNA^{Met}$ as competitors in the Arc1p-tRNA binding reaction. Specifically we utilized the 35 nucleotide minihelix corresponding to the TΨC arm-acceptor stem (acceptor minihelix) and the 19 nucleotide anticodon hairpin minihelix (anticodon minihelix) (Senger *et al.*, 1995). The presence of high molar excess of the anticodon arm in the binding reaction had no effect, suggesting that this part of tRNA is not able to interact with Arc1p. In contrast, the longer T arm-acceptor stem mini-helix partially inhibited the formation of stable Arc1p–tRNA complexes (Figure 6B and C) and, therefore, it may contain part of the binding determinants for Arc1p. These could reside in the TΨC arm as its sequence is

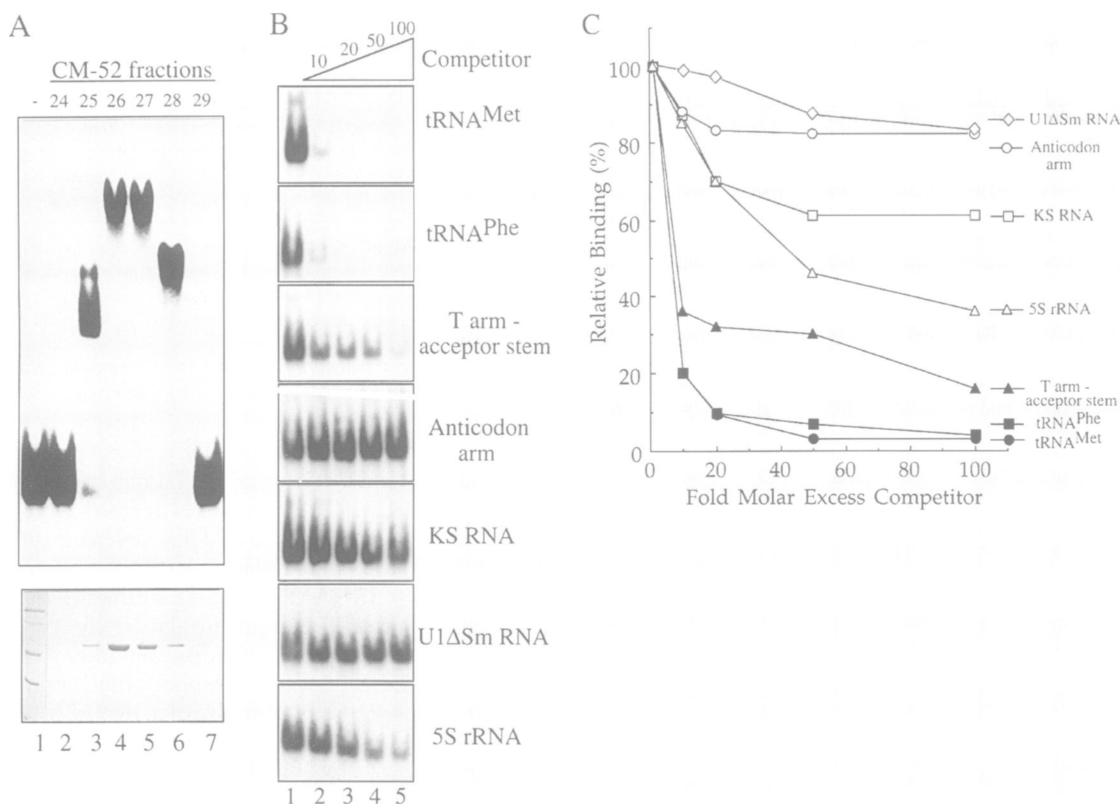

Fig. 6. Recombinant Arc1p binds to tRNA. (**A**) Recombinant Arc1p was applied onto a CM-52 column and bound protein was eluted by a salt gradient. 0.5 µl of each eluted fraction was tested for binding to *in vitro* transcribed and ^{32}P-radiolabelled human initiator $tRNA^{Met}$ (2 ng) using a band shift assay (upper panel) and 1 µl was analysed by SDS–PAGE and Coomassie staining (lower panel). Lane 1, upper panel: no protein added; lane 1, lower panel: molecular weight markers (97, 66, 45 and 31 kDa). (**B**) Recombinant Arc1p (100 ng) was tested in the band shift assay with 1 ng ^{32}P-labelled yeast $tRNA^{Met}$ in the absence of competitor (lane 1) or in the presence of increasing amounts of unlabelled $tRNA^{Met}$, $tRNA^{Phe}$, T arm-acceptor stem, anticodon arm, KS RNA, U1ΔSm snRNA and 5S-ribosomal RNA as indicated. Numbers on top of the figure show the molar excess of the competitor used in each lane. (**C**) Quantification of the experiment shown in (B). The intensity of the shifted band in the absence of competitor is taken as the 100% binding value. ●, $tRNA^{Met}$; ■, $tRNA^{Phe}$; ▲, T arm-acceptor stem; △, 5S-ribosomal RNA; □, KS RNA; ○, anticodon arm; ◇, U1ΔSm snRNA.

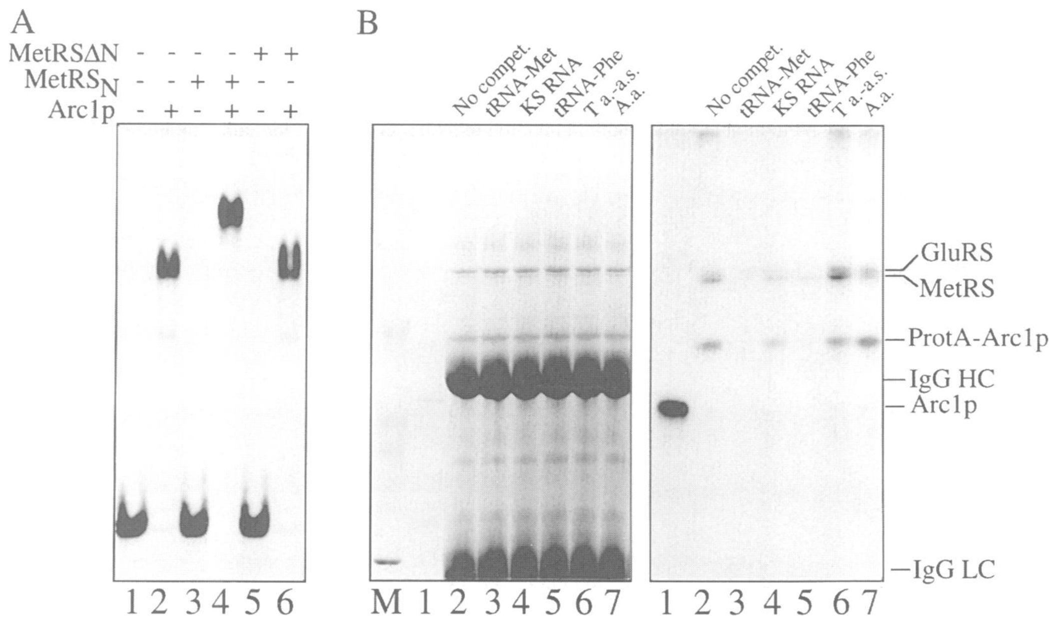

Fig. 7. Arc1p can bind simultaneously to tRNA and aminoacyl-tRNA synthetases. (**A**) Purified yeast full-length $MetRS_N$ or the N-terminally truncated form MetRSΔN are tested alone or in combination with recombinant Arc1p in the band shift assay. Neither $MetRS_N$ (lane 3) or MetRSΔN (lane 5) produce a shift when incubated alone with yeast $tRNA^{Met}$, but $MetRS_N$ causes a super shift of the Arc1p–tRNA complex (lane 4) which is not seen in the case of MetRSΔN (lane 6). (**B**) Recombinant Arc1p (lane 1), and the ProtA–Arc1p/MetRS/GluRS complex bound to IgG–Sepharose beads (lanes 2–7) were UV-irradiated in the presence of 3 ng ^{32}P-labelled yeast $tRNA^{Met}$ and analysed by SDS–PAGE and Coomassie staining (left panel) or autoradiography (right panel). Samples in lanes 3–7 contained a 200-fold molar excess of unlabelled competitor RNA as indicated on top of each lane. Lane M contains molecular weight markers (97, 66, 45 and 31 kDa). T a.-a.s., T arm-acceptor stem; A.a., anticodon arm; HC, heavy chain; LC, light chain.

more conserved between different tRNAs than that of the acceptor arm.

Since recombinant Arc1p can bind to both MetRS and tRNA, we tested whether these two properties are exclusive or not. As shown in Figure 7A, MetRS alone did not form a stable complex with tRNA under the gel-shift assay conditions used (lane 3), but when mixed with Arc1p in the binding reaction it produced a super-shift of the Arc1–tRNA complex (compare lanes 2 and 4), suggesting that MetRS can also associate with the tRNA-bound form of Arc1p. In contrast, no effect was observed when an equal amount of the N-terminally truncated form of MetRS (MetRSΔN), which also does not bind to free Arc1p, was used instead (Figure 7A, compare lanes 2 and 6). To further confirm that Arc1p can associate simultaneously with tRNA and an aminoacyl-tRNA synthetase, we used yeast ProtA–Arc1p in a UV-cross-linking assay. Recombinant Arc1p can be cross-linked efficiently to radiolabelled $tRNA^{Met}$ after UV-irradiation as revealed by polyacrylamide gel electrophoresis and autoradiography (Figure 7B, lane 1). When the IgG–Sepharose beads carrying the MetRS/GluRS/ProtA–Arc1p complex were applied in the same assay, ProtA–Arc1p as well as MetRS and GluRS were cross-linked to tRNA while the abundant chains of IgG were not labelled at all (Figure 7B, lane 2). The cross-link to ProtA–Arc1p was out-competed by an excess of unlabelled yeast $tRNA^{Met}$ or $tRNA^{Phe}$, but not by the KS RNA or the $tRNA^{Met}$ anticodon arm (Figure 7B, lanes 3–5 and 7), reproducing the results obtained from the gel-retardation experiments. The T arm-acceptor stem minihelix, which partially inhibited the formation of recombinant Arc1p–tRNA complexes in the gel-shift assay, had no detectable effect on the cross-linking of ProtA–Arc1p to tRNA (Figure 7B, lane 6), indicating that Arc1p may exhibit higher specificity when in association with MetRS and/or GluRS. In summary, using two different methods we have shown that Arc1p, in free or complexed form, can bind specifically to tRNA (see also Discussion).

Intracellular location of Arc1p

The subcellular distribution of Arc1p was analysed by indirect immunofluorescence using affinity-purified antibodies, raised against *E.coli*-expressed Arc1p. These antibodies specifically recognized a 50 kDa protein on Western blots of total yeast cell extracts which was absent when extracts were derived from the *arc1⁻* strain (data not shown). As shown in Figure 8A, anti-Arc1p antibodies label the cytoplasm of wild-type yeast cells by indirect immunofluorescence, but no signal was seen for example inside the nucleus or vacuole. However, in a significant number of cells, the Arc1p labelling is not homogeneous throughout the cytoplasm but appears more intense close to and around the nuclear periphery. This labelling is specific as cells devoid of Arc1p show no signal at all (data not shown). Similar results were obtained in a double-labelling experiment in which the nuclear pore complexes are decorated with the mouse monoclonal anti-nucleoporin antibody mAb414 (Figure 8B).

Discussion

Since the generation of functional tRNA depends on the nuclear pore-associated protein Los1p (Simos *et al.*, 1996), we sought to identify its interacting components to gain further insight into the various steps of tRNA biogenesis

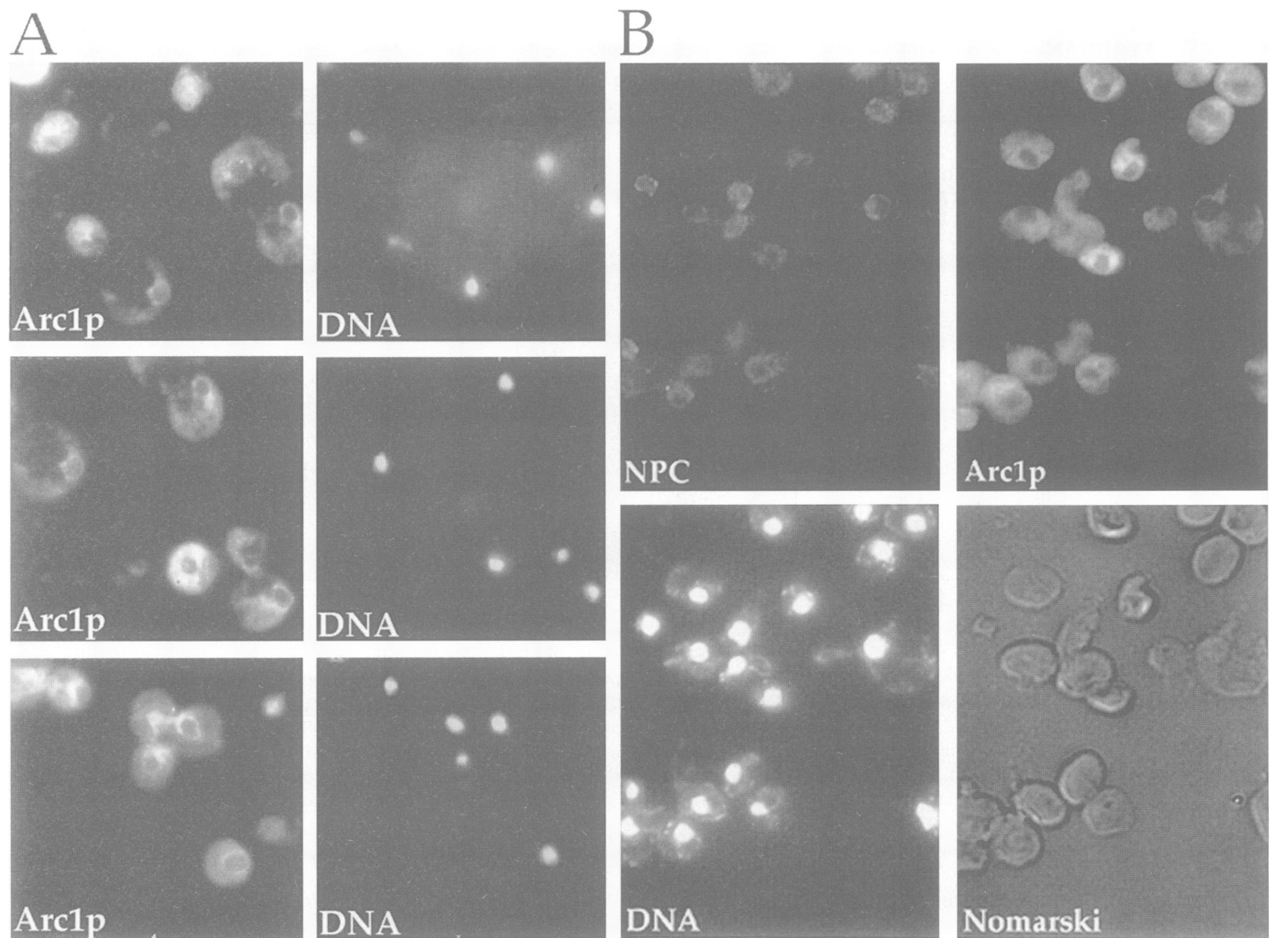

Fig. 8. Intracellular localization of Arc1p. (**A**) Arc1p was localized in wild-type yeast cells by indirect immunofluoresence microscopy using affinity-purified anti-Arc1p antibodies. Panels on the left show antibody staining and panels on the right, the corresponding staining for DNA. (**B**) Double immunofluoresence microscopy in wild-type yeast cells. The nuclear pore complexes (NPC) are labelled with the mouse monoclonal anti-nucleoporin antibody mAb414 and Arc1p as in (A). Cells were also stained for DNA and visualised by Nomarski optics.

and transport. *ARC1* repeatedly appeared in a genetic screen using a disrupted los1$^-$ strain. Although neither *ARC1* nor *LOS1* are essential, strains lacking both genes are not viable. This shows that the functions of Los1p and Arc1p strongly overlap or depend on each other. Clues as to how this interaction occurs may come from the biological function of Arc1. Arc1p is a highly conserved protein that associates physically with two class I amino-acyl-tRNA synthetases, MetRS and GluRS. Moreover, as shown by *in vitro* experiments, Arc1p binds specifically to tRNA and forms a complex with full-length MetRS, thereby increasing the apparent affinity for its cognate tRNA^{Met}. Arc1p is, therefore, the first characterized protein which specifically associates with a eukaryotic aminoacyl-tRNA synthetase and modulates its catalytic properties. Note that the Arc1p-associated proteins were identified though the use of a recently established highly sensitive method which correlates mass spectrometric sequence information with genomic sequence (Mann and Wilm, 1994; Wilm *et al.*, 1996). In this way gene products can quickly be associated with their corresponding genes. Since the complete genome of *Saccharomyces cerevisiae* is now publicly available, the method which we have used here should be very useful for biochemical/functional studies in yeast.

In most of its sequence, Arc1p is homologous to proteins of higher eukaryotes. Strikingly, a 100 amino acid domain is also found conserved in prokaryotes where it is present either as an independent ORF or fused to the catalytic core of the bacterial MetRS (at the C-terminus) and PheRS (at the N-terminus). The eukaryotic MetRS has lost this domain but has acquired an N-terminal extension which, as our results demonstrate, is required for binding to Arc1p. Therefore the yeast MetRS, by associating with Arc1p, is re-connected intermolecularly with the yeast homologue of the prokaryotic C-terminal domain. The evolutionary advantage of this 'cut and re-join' is not yet clear but certainly indicates a unique functional role for this domain. Many other aminoacyl-tRNA synthetases from yeast and higher eukaryotes (Kisselev and Wolfson, 1994), including GluRS from yeast (data not shown), contain N-terminal extensions compared to their pro-karyotic counterparts. Despite the fact that these N-terminal extensions are in several cases not essential for enzymatic function (Mirande, 1991), they may be required *in vivo* for efficient aminoacylation and maximal growth rates by stabilizing the association with Arc1p or other proteins of similar function. Higher eukaryotic MetRS and GluRS are associated together with seven more aminoacyl-tRNA synthetases in a supramolecular

multi-enzyme complex of poorly defined function (Mirande, 1991; Kerjan *et al.*, 1994). Sequencing of the 43 kDa non-enzymatic component of this complex derived from CHO cells (M.Mirande, personal communication), revealed that it is highly homologous to mouse and human EMAP II, and to the C-terminal domain of yeast Arc1p. Therefore, the GluRS/MetRS/Arc1p complex in yeast probably represents an evolutionary intermediate during the formation of larger multi-enzyme complexes.

As shown by two independent RNA-binding assays (bandshift and UV-cross-linking), Arc1p binds specifically to tRNA. Arc1p neither reveals homology to RNA-binding proteins nor possesses sequences that match the RNP1 and RNP2 motifs found in RNA-binding proteins (Mattaj, 1993), resembling in this aspect the aminoacyl-tRNA synthetases, many of which also do not contain a consensus sequence for RNA binding (Schimmel and Ribas de Pouplana, 1995). Interestingly, yeast zuotin, initially identified as a nuclear protein that binds to left-handed Z-DNA (Zhang *et al.*, 1992), has been recently shown also to bind to tRNA (Wilhelm *et al.*, 1994). Zuotin, like Arc1p, contains a lysine/alanine-rich domain with similarity to regions in several histone H1 variants (Zhang *et al.*, 1992). During the course of this work, Arc1p was also identified independently as a protein (termed G4p1) which *in vitro* can bind to quadruplex RNA and DNA, but not to single-stranded or doubled-stranded DNA (Frantz and Gilbert, 1995). Although no clues were offered for the biological function of G41p, these data do not contradict our conclusion that Arc1p is a novel tRNA-binding protein. In the tRNA, the T- and the D-arms are spatially close to each other, forming the elbow of the L-shape and contributing to the central core tertiary structure which resembles that of unimolecular antiparallel quadruplex RNA. Actually, the possibility that the relatively conserved T- and D-loops are part of the Arc1p-binding site is in good agreement with the results of our competition experiments.

The simplest explanation for the effect exerted by Arc1p on the K_m of MetRS for tRNA may be that Arc1p binds to tRNA, which *in vivo* may be coming off the ribosome or exiting the nucleus, and directly transfers it to MetRS. This explanation would be consistent with the recently suggested tRNA channelling model (Negrutskii *et al.*, 1994; Stapulionis and Deutscher, 1995). This model proposes that tRNAs are transferred directly from the aminoacyl-tRNA synthetases to the elongation factors and further on to the ribosomes and back to the aminoacyl-tRNA synthetases without dissociating at any of these steps into the soluble cytosol. It is, therefore, a likely possibility that entry of tRNA into the channelled cytoplasmic cycle is mediated by specialized tRNA-binding proteins, such as Arc1p, that facilitate delivery of tRNA to the aminoacyl-tRNA synthetases, at least in the cases of MetRS and GluRS. Even if transfer of tRNA is not direct, Arc1p would generate a high local concentration of tRNA in the vicinity of the catalytic core of the Arc1p-associated enzymes, thereby facilitating the interaction of aminoacyl-tRNA synthetases with their cognate tRNAs. As suggested by our immunofluoresence microscopy experiments, Arc1p may also target MetRS and GluRS to an intracellular location such as the perinuclear region, at which the cytosolic concentration of tRNA could be highest. Alternative explanations, however, can not be excluded. The mere physical association of Arc1p with the N-terminal domain of MetRS may induce a conformational change in the catalytic core of MetRS which results in higher affinity for tRNA, or Arc1p may exert its effect solely through its binding to tRNA. In the first case, the tRNA-binding property of Arc1p would be redundant and we find the latter case unlikely since Arc1p does not affect the activity of the N-terminally truncated form of MetRS, to which it is also unable to bind. We are in the process of further exploring these possibilities by creating mutants of Arc1p, but the definitive answer would have to come from three-dimensional structural studies involving all interacting components.

We have cloned *ARC1* by its genetic interaction with *LOS1*, a gene which has been initially identified to be involved in tRNA splicing (Hurt *et al.*, 1987; Shen *et al.*, 1993). In our previous work we have shown that Los1p is localized at the nuclear pores and not only interacts functionally with the nuclear pore protein Nsp1p, but also with components of the tRNA biogenesis pathway such as the tRNA modification enzyme Pus1p and the transcription factor subunit Tfc4p (Simos *et al.*, 1996). All this suggested that Los1p may be required for efficient nuclear export of tRNA. The results which we report here are consistent with this model. Since we have not been able to detect a stable physical association between Los1p and Arc1p (G.Simos, K.Hellmuth and E.C.Hurt, unpublished results), the observed synthetic lethality between *los1*$^-$ and *arc1*$^-$ alleles has to be explained in functional terms. One possibility is that, in the absence of Los1p, the rate of nuclear tRNA export is reduced, but the resulting decrease in the concentration of cytoplasmic tRNA can be tolerated by the Arc1p-facilitated interaction of tRNA with aminoacyl-tRNA synthetases, as shown in the case of MetRS. However, if both Los1p and Arc1p are missing from the cell, a reduced amount of cytoplasmic tRNA combined with a decrease in the rate of the $tRNA^{Met}$ aminoacylation will make the available pool of charged $tRNA^{Met}$ limiting for protein synthesis. In an alternative, but not exclusive, model, Los1p, as a component associated with nuclear pores, may help to transfer tRNAs directly from the nuclear pores to the aminoacylation enzymes via Arc1p, without involving diffusion steps. This would again be consistent with the tRNA channelling model and would extend it to the newly synthesized tRNAs that exit the nucleus.

In summary, we have found that two class I aminoacyl-tRNA synthetases in yeast are physically and functionally associated with Arc1p, a protein which binds to tRNA and interacts genetically with the nuclear pore-associated protein Los1p. Taking these results together, we suggest that Arc1p facilitates the delivery of tRNA molecules to the Arc1p-associated aminoacyl-tRNA synthetases. This could be an example of a more general mechanism, i.e. that the high efficiency of tRNA transport processes depends on trapping of tRNA by specialized tRNA-binding proteins and direct uptake by the components of the protein translation machinery. Whether the interaction of tRNA with these proteins is also a trigger for nuclear export remains to be shown, but could be a device which guarantees vectoriality in tRNA transport reactions.

Table II. Yeast strains

Strain	Genotype
RS453	α/**a**, *ade2/ade2, leu2/leu2, ura3/ura3, his3/his3, trp1/trp1*
sl29	α, *los1::HIS3, sl29, ade2, ade3, leu2, ura3, trp1* pHT4467-URA3-ADE3-LOS1
arc1⁻	**a**, *arc1::HIS3, ade2, leu2, ura3, his3, trp1*
los1⁻	α, *los1::HIS3, ade2, leu2, ura3, his3, trp1* (Simos *et al*., 1996)
los1⁻ arc1⁻	α, *los1::HIS3, arc1::HIS3, ade2, leu2, ura3, trp1, his3* pHT4467-URA3-ADE3-LOS1
CH1305	**a**, *ade2, ade3, leu2, ura3, lys2, can1* (Kranz and Holm, 1990)
sl arc1 screening strain	α, *arc1::HIS3, ade2, ade3, leu2, ura3, trp1* pHT4467-URA3-ADE3-ARC1
sl arc1 screening strain	**a**, *arc1::HIS3, ade2, ade3, leu2, ura3, lys2* pHT4467-URA3-ADE3-ARC1
slk12	α, *arc1::HIS3, slk12, ade2, ade3, leu2, ura3, trp1* pHT4467-URA3-ADE3-ARC1
slk84	α, *arc1::HIS3, slk84, ade2, ade3, leu2, ura3, trp1* pHT4467-URA3-ADE3-ARC1
slk117	α, *arc1::HIS3, slk117, ade2, ade3, leu2, ura3, trp1* pHT4467-URA3-ADE3-ARC1
slk88	α, *arc1::HIS3, slk88, ade2, ade3, leu2, ura3, trp1* pHT4467-URA3-ADE3-ARC1
slk125	**a**, *arc1::HIS3, slk125, ade2, ade3, leu2, ura3, lys2* pHT4467-URA3-ADE3-ARC1

Materials and methods

Yeast strains, media, microbiological techniques and plasmids

The yeast strains used in this study are listed in Table II. Yeasts were grown in minimal SDC and rich YPD medium. Sporulation of diploid cells on YPA plates and tetrad analysis were performed according to Wimmer *et al.* (1992). Minimal SDC-medium/plates were supplemented by all amino acids and nutrients except those used for the selection or, if indicated, contained 5-fluoro-orotic acid (5-FOA; CSM medium, BIO101, La Jolla, USA). Genetic manipulations were performed as described by Sherman (1990). The following yeast plasmids were used: pUN100, *ARS/CEN* plasmid with the *LEU2* marker (Elledge and Davis, 1988); pHT4467 was constructed by inserting the ADE3 gene in pRS316, an *ARS/CEN* plasmid with the URA3 marker (Sikorski and Hieter, 1989); pUN100-MES1 was constructed by subcloning into pUN100 a 3.3 kb *Sph*I fragment, carrying the *MES1* gene flanked by the *ADH* promoter and the *ADH* terminator, which was excised from plasmid pMVT1 (Walter *et al*., 1989). Plasmids harbouring the genes for human initiator $tRNA^{Met}$ (Jarmolowski *et al*., 1994), yeast $tRNA^{Met}$ (Senger *et al*., 1992), yeast $tRNA^{Ile}$ (with and without intron) (Szweykowska-Kulinska *et al*., 1994), T arm-acceptor stem and anticodon arm mini-helices (Senger *et al*., 1995) were described before. Plasmids encoding $tRNA^{Phe}$ genes (with and without intron) were a gift from O.Uhlenbeck (Boulder, CO).

Cloning and sequencing of the ARC1 gene

The construction of the screening strain for *los1* synthetic lethals, UV mutagenesis and isolation of the synthetically lethal mutants were described before (Simos *et al*., 1996). By mating of the opposite mating type sl strains, four of the sl mutants were shown to belong to the same complementation group. One of these (sl29) was transformed with a yeast genomic library inserted in pUN100 (described in Wimmer *et al*., 1992), and transformants showing a red/white sectoring phenotype and growth on 5-FOA-containing plates could be obtained. A complementing plasmid, which did not contain *LOS1*, was recovered, and subfragments covering the entire complementing activity were subcloned into pUN100 for DNA sequence analysis. The complementing activity of this plasmid is retained within a 3.5 kb *Bam*HI fragment which was sequenced essentially by primer walking with the dideoxy sequencing method and contains the *ARC1* gene. Comparison of the *ARC1* DNA sequence determined in our laboratory with the recently published DNA encoding G41p (Frantz and Gilbert, 1995) showed that both sequences are allelic, but not completely identical. Accordingly, mismatches were observed also on the level of the amino acid sequences. The accession number of *ARC1* is X95481. The fifth sl mutant in this collection called sl3-B1 was complemented by the *TFC4* gene as reported earlier (Simos *et al*., 1996).

Gene disruption of ARC1 and construction of synthetically lethal mutants by combining los1⁻ and arc1⁻ mutant alleles

Disruption of the *ARC1* gene was done by the one-step gene replacement method (Rothstein, 1983). In this study, the *ARC1* gene was disrupted by inserting a 0.9 kb blunt-ended *Bam*HI fragment containing the *HIS3* gene between the *Nde*I and *Bgl*II sites of the 3.5 kb *Bam*HI fragment, replacing nucleotides –83 to +975 (+1 denotes the start of the ORF of *ARC1*). The disrupted gene was excised and the linear fragments used to transform the diploid strain RS453. HIS^+ transformants with the correct integration of the interrupted gene at the *ARC1* locus were verified by Southern analysis (data not shown). Correct integrants were sporulated and tetrads dissected. A 4:0 segregation for viability and a 2:2 segregation for the *HIS3* gene were found for the *arc1* gene disruption, showing that this gene is not essential for cell viability. To construct a haploid yeast strain in which the disrupted *los1* and *arc1* genes are combined, a los1⁻ mutant harbouring pURA3–LOS1 was mated to the mutant arc1⁻. The resulting heterozygous diploids were sporulated and tetrad analysis was performed. For complete tetrads in which the HIS^+/his^- genotype segregates 2:2, one can predict that the two HIS^+ progeny are *los1::HIS3/arc1::HIS3*. A complete tetrad showing this segregation pattern was analysed in greater detail for the segregation of the *HIS3* and *URA3* markers by plating cells on SDC –his and SDC –ura plates, respectively. The HIS^+ progeny *los1::HIS3/arc1::HIS3* also contained the plasmid pURA3–LOS1; therefore, we could test whether the double combination *los1⁻/arc1⁻* gives synthetic lethality by plating this strain on FOA-containing plates at 30°C. Synthetic lethality was complemented by the presence of either pLEU2–LOS1 or pLEU2–ARC1.

Construction and purification of the ProtA–Arc1p and ProtA–GluRS fusion proteins

Epitope-tagging of Arc1p and GluRS was done by fusing two IgG-binding units from *S.aureus* Protein A to the N-terminal end of each protein. A new *Pst*I restriction site was generated at the ATG codon of *ARC1* by PCR-mediated mutagenesis in order to join the ORF with the *Sac*I–*Pst*I restriction fragment corresponding to two IgG-binding units plus the *NOP1* promoter (P_{Nop1}–ProtA cassette). The full-length *ProtA–ARC1* fusion gene was then subcloned in plasmid pUN100. ProtA–Arc1p was functional since it could complement the synthetic lethal phenotype of sl29 and the slow growth phenotype of strain arc1⁻ (data not shown). The gene for GluRS (AC U32265) was recovered by PCR from total genomic DNA as a 2.6 kb fragment and subcloned into the plasmid pRS315 in frame with the P_{Nop1}–ProtA cassette creating the plasmid pPP1–ProtA–GluRS. Affinity-purification of ProtA–fusion proteins was performed as described in Grandi *et al.* (1993) with minor modifications. 3 g of spheroplasted cells (arc1⁻ carrying pUN100–ProtA–Arc1) were lysed in 60 ml of lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris–HCl, pH 8.0) supplemented with protease inhibitors. The cleared lysate was passed through a IgG–Sepharose column and bound material was eluted by lowering the pH to 3.4. In a separate experiment, the material bound to the column was eluted stepwise by increasing concentrations of $MgCl_2$ (1, 2 and 4 M $MgCl_2$ in 50 mM Tris–HCl, pH 7.4). For affinity-purification of ProtA–GluRS, 3 g of cells (RS453 haploid or arc1⁻ carrying pPP1–ProtA–GluRS) were lysed in 35 ml 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 50 mM NaP_i, pH 7.5.

Determination of protein sequences with mass spectroscopy

Proteins separated by PAGE were in-gel digested with trypsin and purified as described (Shevchenko *et al*., 1996; Wilm *et al*., 1996). The recovered, unseparated peptide mixture was electrosprayed with a nano electrospray ion source (Wilm and Mann, 1996) and sequenced by triple quadrupole mass spectrometry as explained in Mann and Wilm (1995). Peptide Sequence Tags (Mann and Wilm, 1994) were searched in a comprehensive, non-redundant database (nrdb, maintained by C.Sander,

EMBL) of 180 000 entries using the program PeptideSearch. No restrictions on mass range or origin of species were applied in the database searches.

Expression of Arc1p in E.coli, purification of recombinant protein and generation of antibodies

The *ARC1* ORF was amplified by PCR using two primers that created a *Xho*I restriction site at the ATG start codon and a *Mlu*I restriction site in the 3′-untranslated region of the gene. This manipulation allowed cloning of the ORF into a modified pET (pET–HIS6/pET8c; Schlaich and Hurt, 1995) vector previously cut with *Xho*I–*Mlu*I and created an in-frame fusion protein of six histidine residues joined by a spacer Ser–Ser dipeptide to the amino acid immediately after the start methionine. The vector containing the fusion gene was transformed into *E.coli* BL21 cells. Induction of expression and purification of the Arc1p fusion protein were performed as previously described by Simos *et al.* (1996). The recombinant protein was dialysed against PBS and was used to immunize rabbits (100 μg per injection). Antibodies from positive sera were affinity-purified using recombinant Arc1p coupled to Affi-Gel 10 (Bio-Rad). In order to test tRNA binding, the recombinant Arc1p was further purified by ion-exchange chromatography. 27 mg of recombinant Arc1p were dialysed against 20 mM Tris–HCl, 5 mM $MgCl_2$, 1 mM DTT, pH 7.5, and passed through a CM-52 (Whatman) column equilibrated in the same buffer. Approximately 50% of the protein was bound to the column and was subsequently eluted by applying a gradient of 0–500 mM NaCl in the equilibration buffer.

Isolation of synthetic lethal mutants of the arc1::HIS3 allele

To construct a *arc1::HIS3* strain with the *ade2/ade3* markers which are required for the red/white colony sectoring assay (Wimmer *et al.*, 1992), the $arc1^-$ strain was mated to CH1305 (see Table II). The derived diploid strain was sporulated, and haploid progeny with both *arc1::HIS3* and *ade2/ade3* were selected after tetrad analysis and transformed with pHT4467–URA3–ADE3–ARC1. The resulting screening strain for *arc1* synthetic lethals which was available in both mating types (see Table II) formed red/white sectoring colonies on YPD plates. UV-mutagenesis of the sl screening strain was carried out essentially as described (Wimmer *et al.*, 1992), and 30 000 surviving colonies were screened for a stable red, non-sectoring phenotype at 30°C and no growth on 5-FOA plates. Putative sl mutants were finally transformed with pUN100–LEU2–ARC1 and six of them regained a red/white sectoring phenotype and could grow on 5-FOA-containing plates. Slk117 could be complemented by pUN100–MES1, slk88 and slk125 by pPP1–ProtA–GluRS, slk12 and slk84 by pUN100–LOS1.

Aminoacylation assays

Methionyl-tRNA synthetase activity was measured in crude cell extracts, obtained by mechanical breakage using glass beads or using purified MetRS (Despons *et al.*, 1992) in the absence or presence of recombinant Arc1p. In the latter case, MetRS and Arc1p (5 μM each) were preincubated at 25°C in 5-fold aminoacylation buffer (700 mM Tris–HCl, pH 7.8, 50 mM DTT, 500 μg/ml BSA) for 10 min before dilution in 1-fold aminoacylation buffer. The final concentration of MetRS varied from 10 nM to 20 nM. Similar experiments were repeated with a N-terminal-truncated MetRS form (MetRSΔ6-185; Walter *et al.*, 1989). Aminoacylation assays were carried out at 25°C as described by Senger *et al.* (1992). K_m for $tRNA^{Met}$ was measured using purified yeast initiator $tRNA^{Met}$ (a gift from G.Keith, Strasbourg). The concentration of tRNA varied from 1 to 40 μM in the absence and from 0. 1 to 1 μM in the presence of Arcp1.

Protein and tRNA binding assays

To detect *in vitro* binding of Arc1p to MetRS, ~1 μg of MetRS (full-length $MetRS_N$ or truncated MetRSΔ6-185) was incubated with 1 μg of recombinant Arc1p in 100 μl 0.1% Triton-X 100, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml BSA, 50 mM Tris–HCl, pH 7.5, at room temperature for 15 min and Arc1p was immunoprecipitated by the addition of 5 μg affinity-purified anti-Arc1p antibodies. For control, MetRS was incubated alone. For binding to tRNA, 100–200 ng of purified recombinant Arc1p was incubated for 30 min at 30°C with 1–2 ng of *in vitro*-transcribed ^{32}P-labelled tRNA (100 000 c.p.m./ng) in 20 μl of 20 mM HEPES, pH 7.5, 150 mM NaCl, 7.5 mM $MgCl_2$, 10% glycerol, 0.2 U/μl RNasin (Promega) and RNA–protein complexes were detected by electrophoresis on 5% non-denaturing polyacrylamide/0.5× TBE gels (2.5 h, 120 V) followed by autoradiography. RNA–protein complexes were quantitated by the use of a phosphorimager (Molecular Dynamics, USA) supplied with the Image Quant software. For the cross-linking experiments, 1 μg of recombinant Arc1p or 5 μl of IgG–Sepharose beads carrying the ProtA–Arc1p/MetRS/GluRS complex were mixed with 3 ng of ^{32}P-labelled tRNA in 10 μl of 20 mM HEPES, pH 7.5, 7.5 mM $MgCl_2$, 10% glycerol, 0.2 U/μl RNasin (Promega), 0.1 mg/ml BSA, incubated for 20 min at 30°C and UV-irradiated for 10 min on ice in a UV Stratalinker 1800 (Stratagene). The RNA was digested for 30 min at 37°C with 10 U of RNase T1 and 15 μg of RNase A before addition of sample buffer, SDS–PAGE and autoradiography. In all competition studies, the unlabelled RNAs were added to the reaction before the addition of the hot probe. T7 transcription in presence or absence of [α-^{32}P]UTP was performed as previously described (Simos *et al.*, 1996) and the RNAs were purified by denaturing polyacrylamide gel electrophoresis, elution and ethanol precipitation and quantitated by absorption at 260 nm or ethidium bromide staining. Purified transcripts were renatured before use by heating at 90°C for 1 min and cooling at room temperature for 5 min. 5S-ribosomal RNA from *E.coli* was purchased from Boehringer-Mannheim and U1ΔSm snRNA (Jarmolowski *et al.*, 1994) was a generous gift from Dr E.Izaurralde (EMBL, Heidelberg).

Miscellaneous

DNA manipulations (restriction analysis, end-filling reactions, ligations, PCR amplifications, etc.) were done essentially according to Maniatis *et al.* (1982). Isolation of total yeast DNA and Southern analysis was performed essentially as described by Sherman *et al.* (1986). Preparation of protein extracts, SDS–PAGE, Western blotting, immunoprecipitation and indirect immunofluorescence were performed as described by Grandi *et al.* (1993).

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