The EMBO Journal Vol.17 No.16 pp.4780–4789, 1998

Tad1p, a yeast tRNA-specific adenosine deaminase, is related to the mammalian pre-mRNA editing enzymes ADAR1 and ADAR2

André Gerber, Henri Grosjean[1], Thorsten Melcher[2] and Walter Keller[3]

Department of Cell Biology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland, [1]Laboratory of Structural Enzymology and Biochemistry, CNRS, F-91198 Gif-sur-Yvette, France and [2]Department of Molecular Neuroscience, Max-Planck-Institute for Medical Research, D-69120 Heidelberg, Germany

[3]Corresponding author
e-mail: Keller2@ubaclu.unibas.ch

We have identified an RNA-specific adenosine deaminase (termed Tad1p/scADAT1) from *Saccharomyces cerevisiae* that selectively converts adenosine at position 37 of eukaryotic tRNA^{Ala} to inosine. The activity of purified recombinant Tad1p depends on the conformation of its tRNA substrate and the enzyme was found to be inactive on all other types of RNA tested. Mutant strains in which the *TAD1* gene is disrupted are viable but lack Tad1p enzyme activity and their tRNA^{Ala} is not modified at position A_{37}. Transformation of the mutant cells with the *TAD1* gene restored enzyme activity. Tad1p has significant sequence similarity with the mammalian editing enzymes which act on specific precursor-mRNAs and on long double-stranded RNA. These findings suggest an evolutionary link between pre-mRNA editing and tRNA modification.

Keywords: adenosine deaminase/inosine/RNA editing/ tRNA^{Ala}/yeast

Introduction

The nucleoside inosine (I) was first detected in cytoplasmic tRNAs (Holley *et al*., 1965) and has recently been observed in viral and nuclear encoded RNA transcripts (reviewed in Bass, 1997; O'Connell, 1997; Rueter and Emeson, 1998; see also Paul and Bass, 1998). In both types of RNAs, messenger RNA precursors (pre-mRNAs) and tRNAs, I is formed by hydrolytic deamination of adenosine (A) (Grosjean *et al*., 1996a; Bass, 1997). Because I is read as guanosine (G) by the translational machinery (Basilio *et al*., 1962), the site-specific conversion of A to I in pre-mRNAs can alter single codons and change their amino acid specificity. The resulting variation in protein sequence can profoundly affect biological function. In general, this and other types of RNA editing events are believed to play an important role in the generation of genetic diversity in eukaryotes (reviewed in Kable *et al*., 1997; Smith *et al*., 1997).

In eukaryotic tRNA^{Ala}, I is present at the first anticodon position (position 34) and as the derivative N^1-methylinosine (m^1I) at position 37 (3′ adjacent to the anticodon). I_{34} is found in eight cytoplasmic tRNAs of eukaryotic organisms, whereas in prokaryotes and plant chloroplasts only tRNA^{Arg2} contains I_{34} (reviewed in Björk, 1995). In contrast, m^1I_{37} is unique to eukaryotic tRNA^{Ala} and to date has not been detected in any prokaryotic tRNA. However, in archaeal tRNAs, m^1I is frequently found at position 57, in the middle of the Ψ-stem (Grosjean *et al*., 1996a; see also Sprinzl *et al*., 1998). The synthesis of m^1I_{37} in eukaryotic tRNA^{Ala} occurs by a two-step enzymatic process. The first step is the deamination of A_{37} to I_{37} followed by methylation of I_{37} by a *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase (Grosjean *et al*., 1996a; H.Grosjean, unpublished results). The enzymes involved in the formation of I or m^1I in tRNAs have not yet been identified except for the partial purification and initial characterization of the yeast tRNA:A_{34} deaminase (Auxilien *et al*., 1996).

In contrast, the enzymes responsible for the deamination of A to I in pre-mRNAs, the adenosine deaminases acting on RNA (ADARs) have been extensively studied (reviewed in Bass *et al*., 1997; Maas *et al*., 1997; Rueter and Emeson, 1998). To date, three sequence-related ADAR or ADAR-like enzymes have been cloned and characterized from different sources: ADAR1 (Kim *et al*., 1994; O'Connell *et al*., 1995; Hough and Bass, 1997; Liu *et al*., 1997), ADAR2 (Melcher *et al*., 1996b; Gerber *et al*., 1997; Lai *et al*., 1997; Mittaz *et al*., 1997) and RNA specific editase 2 (RED2; Melcher *et al*., 1996a). These members of the ADAR enzyme family contain two or three double-stranded RNA binding domains (dsRBD; St Johnston *et al*., 1992) and a catalytic deaminase domain (Figure 1A). Moreover, several alternatively spliced variants of human (h) ADAR1 and hADAR2 exist (Gerber *et al*., 1997; Lai *et al*., 1997; Liu *et al*., 1997). Of the four variants of hADAR2 (a,b,c,d), hADAR2b differs from hADAR2a by the insertion of a 40 amino acid alu-J cassette within the deaminase domain.

Mammalian ADAR1 and ADAR2 catalyze the site-specific deamination of adenosines in pre-mRNAs encoding subunits of glutamate-gated ion channels (GluR) expressed in the brain (Dabiri *et al*., 1996; Maas *et al*., 1996; Melcher *et al*., 1996b; O'Connell *et al*., 1997). These editing events have been correlated with physiological effects on calcium permeability and the kinetics of the ion channels (reviewed in Seeburg, 1996). In addition, ADAR1 and/or ADAR2 have been implicated in the editing of mammalian $5HT_{2C}$ serotonin receptor mRNA (Burns *et al*., 1997) and hepatitis delta virus (HDV) antigenomic RNA (Polson *et al*., 1996, 1998). In all of these cases, editing requires a dsRNA structure which is formed by intramolecular base pairing between exonic and intronic sequences in pre-mRNAs (Higuchi *et al*., 1993; Lai *et al*., 1995; Maas *et al*., 1996). Unlike ADAR1 and ADAR2, recombinant RED2 fails to deaminate adenosines in extended dsRNA, and no specific RNA substrate has been found yet (Melcher *et al*., 1996a). The content

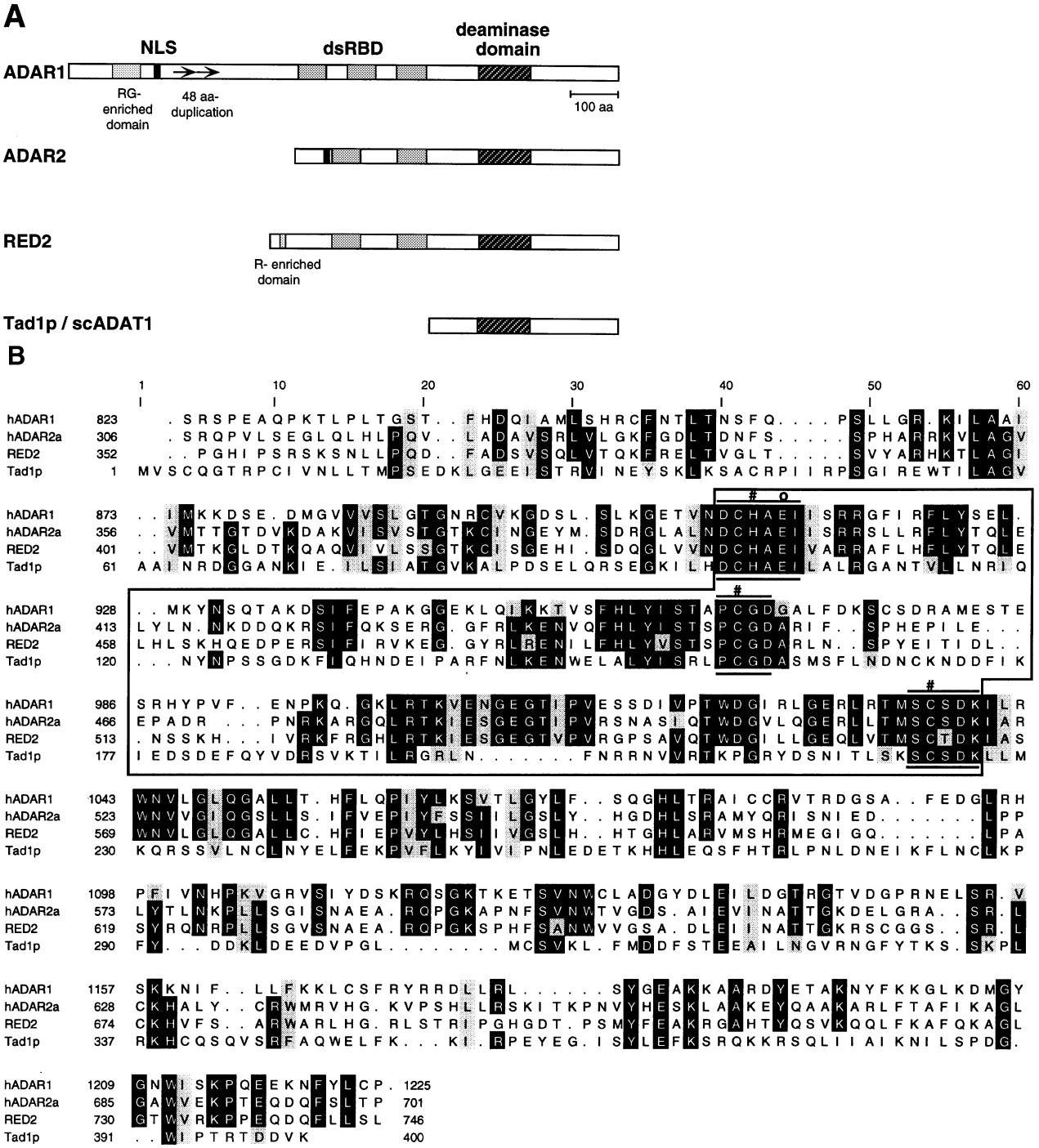

Fig. 1. (**A**) Protein domain structure of mammalian adenosine deaminases that act on RNA (ADARs) and the homologous *S.cerevisiae* protein Tad1p/scADAT1. ADAR1 and ADAR2 contain bipartite nuclear localization signals (NLS, black box). Double-stranded RNA-binding domains (dsRBDs) are depicted as grey boxes. The catalytic deaminase domain is represented by a cross-hatched box. (**B**) Multiple sequence alignment of members of the ADAR family and Tad1p. Highly conserved residues (>75%) are depicted within a black frame, similar amino acids which are conserved in all four proteins are boxed in grey. The deaminase domain (boxed) is characterized by conserved motifs (underlined) containing three putative Zn^{2+}-chelating residues (#) and a proton-transferring amino acid (o). The DDBJ/EMBL/GenBank accession Nos of the sequences are as follows: hADAR1, U10439; hADAR2a, U82120; RED2, U74586, Tad1p, AJ007297. The alignment was generated with the GCG software programs LINEUP (manual alignment) and PILEUP (computer alignment) with a gap weight of 3.0 (Devereux *et al*., 1984). The C-terminal part of yeast Tad1p (from aa 259) was further aligned manually to the other three sequences.

of I in mRNAs has been measured in a variety of mammalian tissues and revealed the highest amounts in the brain (one I in 17 000 ribonucleotides; Paul and Bass, 1998); this suggests that additional targets for these editing enzymes exist.

In the search for new members of the ADAR enzyme family we have cloned and characterized a sequence-related enzyme from *Saccharomyces cerevisiae*. Interestingly, the yeast enzyme Tad1p does not form I in pre-mRNA but is responsible for the formation of I at

position 37 in yeast $tRNA^{Ala}$. This is, therefore, the first tRNA-specific adenosine deaminase [Tad1p alias scADAT1 (adenosine deaminase acting on tRNA 1)] that has been cloned and its gene will further be referred to as *TAD1*. Moreover, this finding provides a model for the evolution of the A to I mRNA editing enzymes found in higher eukaryotes.

Results

Cloning of the TAD1 gene and heterologous expression in Pichia pastoris

The analysis of open reading frames (ORFs) located on yeast *Saccharomyces cerevisiae* chromosome VII revealed an ORF (YGL243w/HRA400) which is 22% identical (46% similar) to the C-terminal part of the mammalian RNA editing enzymes ADAR1 and ADAR2 (Vandenbol *et al*., 1995). The homologous section contains the catalytic core of ADAR1 and ADAR2 (boxed in Figure 1B) which is characterized by three conserved deaminase motifs, DCHAE, PCGD and SCSDK, containing putative Zn^{2+}-chelating residues and a proton transferring glutamate (reviewed in Smith *et al*., 1997; see also Carter, 1995). Importantly, these motifs are conserved in the yeast protein, but the similarity of the yeast protein to mammalian ADARs extends beyond this putative deaminase domain. Unlike ADAR1, ADAR2 and RED2, which all contain dsRBDs upstream of the deaminase domain (Figure 1A), neither a dsRBD nor any other of the known types of RNA binding domains could be seen in the HRA400 sequence. We tested *S.cerevisiae* S100 extract for its ability to convert A to I in long dsRNA, an assay which is routinely used to measure ADAR1/2 activity (O'Connell and Keller, 1994), but did not detect any activity (results not shown). This indicates that yeast does not contain any classical dsRNA-specific adenosine deaminases. We therefore explored the possibility that partially dsRNAs, such as tRNAs, may be the natural substrate for HRA400p.

The ORF YGL243w was amplified by the polymerase chain reaction (PCR) from *S.cerevisiae* genomic DNA. Since we previously found that the methylotrophic yeast *Pichia pastoris* is a suitable organism for the production of active recombinant ADARs (Gerber *et al*., 1997; O'Connell *et al*., 1998), the coding sequence of *TAD1* was subcloned into a *P.pastoris* expression vector that was engineered to encode a 5′-FLAG- and a 3′-histidine hexamer epitope (O'Connell *et al*., 1998). Recombinant Tad1p was purified to homogeneity from a protein expressing clone (Figure 2A and B) and ~50 µg of pure (>99.5%) and highly active Tad1p was obtained from 43 g of yeast cells. Recombinant Tad1p has a Stokes' radius of 32.5 Å which corresponds to a molecular mass of 50 kDa.

Tad1p converts A_{37} to I_{37} in $tRNA^{Ala}$

During purification of the recombinant yeast protein, aliquots of the column fractions were assayed for their ability to convert A to I either at positions 34 or 37 in synthetic yeast $tRNA^{Ala}$. Peak fractions of the final column containing pure recombinant Tad1p can form <0.9 mol I/mol $tRNA^{Ala}$ in the absence of added cofactors (Figure 2). Note that I and m^1I would comigrate in the one-dimensional TLC as shown in Figure 2C. Therefore, the identity of I was verified by two-dimensional (2D) TLC which separates I from m^1I (Silberklang *et al*., 1979). We found no m^1I in yeast $tRNA^{Ala}$ after incubation with pure Tad1p and SAM, as analyzed by 2D-TLC (results not shown). This indicates that Tad1p has no SAM-dependent methyltransferase activity *in vitro*.

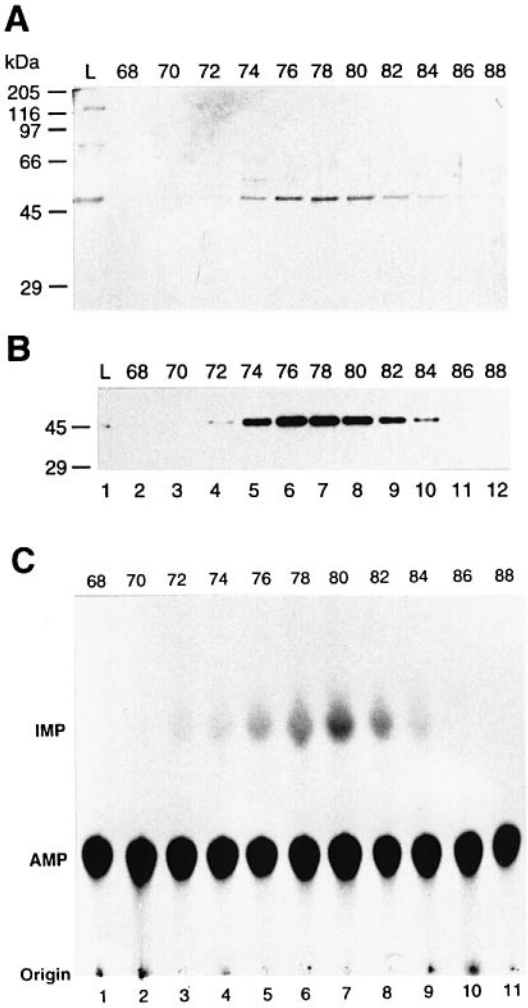

Fig. 2. Gel-filtration chromatography of recombinant Tad1p. (**A**) Aliquots (15 µl) of the final column were electrophoresed on a 10% SDS–polyacrylamide gel, and proteins were visualized by silver staining. Fraction numbers are indicated at the top. Molecular masses are indicated in kDa on the left. (**B**) Immunoblot analysis with a mouse anti-FLAG M2 monoclonal antibody (1:5000). (**C**) Conversion of A_{37} to I in yeast $tRNA^{Ala}$. Two µl of each fraction was incubated with 200 fmol [^{33}P]ATP-labelled transcripts of yeast $tRNA^{Ala}$ ($A_{34}G$) for 45 min at 30°C. After ethanol precipitation the RNA was digested with P1 nuclease and the reaction products were separated by one-dimensional TLC. The spots at the bottom are the origin; the spots in the middle correspond to AMP; the spots at the top to IMP.

To determine the location of I formed in yeast $tRNA^{Ala}$ by Tad1p, RT–PCR was performed on modification products, and the cDNAs were sequenced in both directions. The result depicted in Figure 3A demonstrates that recombinant Tad1p specifically deaminates A_{37}, but not A_{34} in yeast $tRNA^{Ala}$. The reaction is almost complete (80–90% of A_{37}–I_{37} conversion). This correlates well with the 0.9 mol I/mol tRNA measured by TLC.

tRNA editing is strictly magnesium dependent with an optimal concentration between 2–3 mM (data not shown).

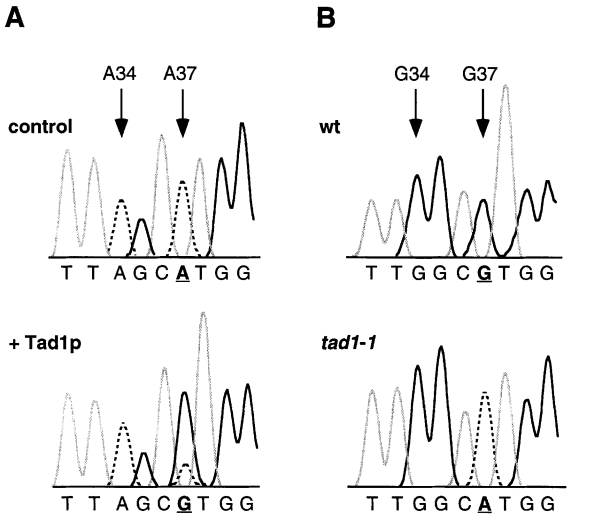

Fig. 3. Sequence analysis of edited yeast $tRNA^{Ala}$. Only the region surrounding A_{34} and A_{37} in the anticodon loop is depicted. Positions 34 and 37 are marked by an arrow. The edited nucleotides are in bold and underlined. Adenosine peaks are indicated by a dashed line and guanosine peaks are bold. Since inosine prefers to base pair with C, I is represented as G. (**A**) *In vitro* transcribed yeast $tRNA^{Ala}$ was incubated with 0.2 ng purified recombinant Tad1p for 1 h at 30°C. As a control, no enzyme was added to the reaction. Fifty fmol of the tRNA was reverse transcribed (RT), amplified by PCR and the products were sequenced. (**B**) tRNAs were isolated from a wt strain and from a *tad1-1* mutant strain. $tRNA^{Ala}$ was amplified by RT–PCR and subcloned. Individual clones were sequenced and one representative is depicted.

In the absence of Mg^{2+} ions no A_{37}–I_{37} conversion occurred whereas addition of high amounts (>10 mM) strongly reduced the yield of I_{37} (<10%). A similar dependence on Mg^{2+} concentration was previously reported for the yeast tRNA:A_{34} deaminase activity (Auxilien *et al*., 1996) and was explained by the dependence of the enzyme on the 3D architecture of the tRNA substrate rather than by a direct involvement of Mg^{2+} in catalysis. The KCl optimum for A_{37}–I_{37} conversion by Tad1p is 25 mM and high salt concentrations (>100 mM KCl) inhibited the reaction. The pH optimum is between 7 and 8.

In order to test whether the editing enzymes ADAR1 or ADAR2 can also deaminate selected As in tRNAs, we performed the same *in vitro* assays as above with recombinant hADAR1 and hADAR2 (O'Connell *et al*., 1998). Even in the presence of 50–100 ng of purified hADAR1 or hADAR2 per assay, compared to 0.2 ng of Tad1p, we were unable to detect any I formation in yeast $tRNA^{Ala}$, nor in $tRNA^{Ala}$ from the silkworm *Bombyx mori* (data not shown). This tRNA is 90% identical to the human form and is a substrate for human Tad1p activity, as measured with HeLa nuclear extract (A.Gerber and W.Keller, unpublished results). Conversely, we assayed pure recombinant Tad1p for its ability to convert A to I in extended dsRNA (O'Connell and Keller, 1994) and at the Q/R, R/G and so-called hotspot 1 site in pre-mRNA of GluR-B (Melcher *et al*., 1995), substrates edited by ADAR1 and/or ADAR2. No conversion of A to I was detected (data not shown), demonstrating that pre-mRNA and extended dsRNA are not used as substrates by Tad1p *in vitro*.

Characterization of tad1 mutants

Two *TAD1* disruption strains were constructed. In one strain (AG1), amino acid residues 48–186 of the coding sequence YGL243w were replaced with the selectable *TRP1* gene (*tad1-1*). A second strain (AG2) was constructed by the insertion of the *TRP1* marker at residue 186 in the ORF of *TAD1* (*tad1-2*). Correct replacement or insertion of the *TRP1* marker was confirmed by PCR. Sporulation of the diploids and dissection of tetrads yielded four viable spores, which all grew normally and to the same size (data not shown). Cells grown from two of the four viable spores were Trp^+ indicating that *TAD1* is not essential for cell viability. Moreover, when tryptophan prototrophs (haploids AG3, AG4) were grown in yeast extract, peptone, dextrose (YPD) or minimal medium at 30°C, no significant differences in the doubling times could be measured compared with the wild-type (wt) haploid cells (1n, BMA41). These results indicate that no significant slow growth phenotype can be attributed to *tad1* mutants at 30°C.

To confirm that A_{37} in $tRNA^{Ala}$ is the target of the *TAD1* gene product *in vivo*, total RNA was isolated from the *tad1-1* mutant cells (AG3) and from wt control cells (1n, BMA41) respectively. RT–PCR was performed on total RNA with $tRNA^{Ala}$ specific primers, the cDNAs were subcloned and 16 different clones derived from the *tad1-1* strain as well as six clones from the wt strain were sequenced. All clones derived from the *tad1-1* mutant contained guanosine at position 34 (G_{34}) and A at position 37 (A_{37}) (Figure 3B). These results demonstrate unambiguously that Tad1p is involved in the conversion of A_{37} to I_{37} but not in the conversion of A_{34} to I_{34} in $tRNA^{Ala}$ *in vivo*. Since the absence of Tad1p *in vivo* leads to the complete absence of the I_{37} modification in the $tRNA^{Ala}$, we can conclude that there is no redundant activity in yeast which can substitute for Tad1p function. The clones derived from the wt strain contained G_{34} and G_{37} (3 clones) or thymidine-37 (T_{37}; 3 clones; Figure 3B). The presence of G_{37} or T_{37} in these RT–PCR products suggests the presence of a modified base, most probably m^1I. As has been described for m^1A (Kröger and Singer, 1979), m^1I may base pair with reduced specificity in the RT reaction, explaining the occurrence of T_{37} in the PCR product. *In vitro*, the order of modification has been shown to be A_{37} to I_{37}, followed by methylation to m^1I_{37} (Grosjean *et al*., 1996a; H.Grosjean, unpublished results). However, *in vivo*, the sequential order of modification has not yet been confirmed.

To complement the *in vivo* results, extracts from wt and *tad1-1* mutant cells were tested for their ability to modify $tRNA^{Ala}$ transcripts *in vitro*. Figure 4 shows that an extract of the *tad1-1* mutant is unable to form I_{37} in $tRNA^{Ala}$ of *S.cerevisiae* and *B.mori* (lanes 5 and 6), whereas the formation of I_{34} is not affected (lanes 4 and 7). Formation of I_{37} was restored after transformation of yeast *tad1-1* mutant cells with centromeric vectors bearing the ORF of *TAD1* under the control of its own promoter and terminator sequences (pFL38-YDr13; strain AG5; lanes 8–11) or under the control of an inducible galactose promoter (pGalΔTrp1–Tad1; strain AG6; lanes 12 and 13).

Substrate specificity of Tad1p

We investigated the substrate specificity of recombinant Tad1p by comparing its activity on different tRNAs. The

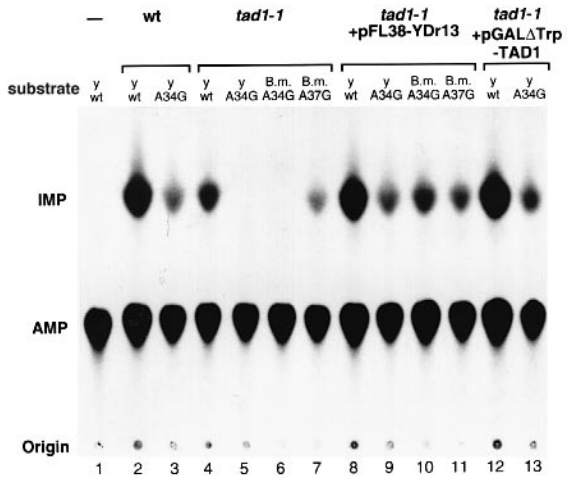

Fig. 4. tRNA-specific adenosine deaminase assay. Different ^{33}P-labelled alanine tRNAs were incubated with yeast cell extracts. Lane 1: no extract; lanes 2 and 3: wild-type extract ; lanes 4–7: *tad1-1* mutant extracts. The mutants can be rescued with either a genomic fragment including *TAD1* promoter and terminator sequences (pFL38-YDr13, lanes 8–11), or by placing *TAD1* under the control of a galactose promoter (pGalΔTrp–TAD1, lanes 12 and 13). Abbreviations: y, yeast; B.m., *B.mori*; A34G, mutant tRNA containing G_{34} instead of A_{34}; $A_{37}G$, G_{37} instead of A_{37}.

activity of yeast extract was also assayed to measure the formation of both, I_{34} and I_{37}. In the assay, Tad1p or S100 extracts were incubated with synthetic tRNA under standard reaction conditions and the RNA was subsequently digested with P1-nuclease. The mononucleotides were separated by one-dimensional TLC and quantified with a PhosphorImager (O'Connell and Keller, 1994; Auxilien *et al*., 1996). The results listed in Table I can be summarized as follows. First, Tad1p deaminates A_{37} in wt $tRNA^{Ala}$ from yeast and *B.mori* (which differs in 19 nt from its yeast counterpart over the total length of 76 nt), but the reaction with the latter substrate is less efficient (0.3–0.4 mol I/mol tRNA). On the other hand, the enzyme can not form I_{37} in a prokaryotic $tRNA^{Ala}$ from *Escherichia coli*. In nature, this tRNA does not contain I, but the tRNA contains A_{37} that could potentially be deaminated. Secondly, mutant *B.mori* $tRNA^{Ala}$ (A37G), in which A_{37} was changed to G, is not a substrate for Tad1p, while in mutant *B.mori* $tRNA^{Ala}$ (A34G), where A_{34} was mutated to G_{34}, conversion of A_{37} to I_{37} could be detected. This demonstrates that I or G at position 34 does not influence the formation of I_{37}. Thirdly, removing one base from the 7 nt anticodon loop in yeast $tRNA^{Ala}$ (mut ΔU33) abolishes both A_{34} and A_{37} conversion to I_{34} and I_{37}, respectively. Thus, as was shown for the yeast tRNA:A_{34} deaminase activity (Auxilien *et al*., 1996), the formation of I_{37} by Tad1p is dependent on the correct anticodon loop size. Fourthly, mutations that possibly affect the 3D architecture of the yeast or *B.mori* $tRNA^{Ala}$ have drastic consequences on I_{37} formation catalyzed by recombinant Tad1p. Indeed, deletion of U13 in the yeast $tRNA^{Ala}$ (mut A34G+ΔU13) disrupting the U_{13}–G_{22} base pair in the narrow groove of the D-stem helix and possibly also the triple interaction with residue 46 (G_{46}; reviewed in Dirheimer *et al*., 1995) abolishes I_{37} formation. Likewise, mutation of U_8 to C_8 in the *B.mori* $tRNA^{Ala}$ (mut U8C) which most likely disrupts the conserved tertiary interaction between U_8 and A_{14}, and the mutation of A_{48} to C_{48} affecting the so-called Levitt tertiary base pair (Dirheimer *et al*., 1995; Grosjean *et al*., 1996b) also drastically reduced I_{37} formation. The residual I formed in these two *B.mori* tRNA mutants by incubation with yeast extracts can probably be assigned to the formation of I_{34} by the tRNA:A_{34} deaminase; it is also possible that another factor in the extract enables Tad1p to recognize and modify this mutated tRNA. It was previously shown that variants of yeast $tRNA^{Asp}$ are substrates for the partially purified tRNA:A_{34} deaminase, although the wt $tRNA^{Asp}$ contains no inosine (Auxilien *et al*., 1996). Our results confirmed these findings by incubating the $tRNA^{Asp}$ mutants G37A, GU3435AA and GU3435AA/G37A with yeast S100 extracts. In contrast, these substrates were totally inefficient for Tad1p activity. Furthermore, a variant of yeast $tRNA^{Asp}$, in which the entire anticodon stem–loop was replaced by that of yeast $tRNA^{Ala}$ (mut AC–loop $tRNA^{Ala}$) is a good substrate for the tRNA:A_{34} deaminase present in the yeast S100 extract, but is totally inefficient as a substrate for Tad1p.

The K_m and turnover number (k_{cat}) of Tad1p were determined on synthetic yeast $tRNA^{Ala}$. A plot of the reaction velocity against substrate concentration yielded a hyperbolic curve and Michaelis–Menten kinetics were anticipated. An apparent K_m of 6 nM (± 4 nM) and V_{max} = 0.22 nM/min (± 0.03) was determined and is an average of three independent measurements (results not shown). This K_m value is in the same order of magnitude as that found for the *S.cerevisiae* tRNA:A_{34} deaminase activity (K_m = 2.3 nM), determined with partially purified enzyme and the natural substrate yeast $tRNA^{Ser}$ (Auxilien *et al*., 1996). From the protein concentration in the assays (0.052 nM) and the measured V_{max}, a k_{cat} of 4.23/min was calculated. Therefore, Tad1p needs ~15 s to recognize, deaminate and release one tRNA molecule under *in vitro* conditions.

Discussion

A new RNA deaminase lacking dsRBDs

In our search for new members of the ADAR enzyme family we have cloned and characterized a yeast RNA deaminase. The homology between the *S.cerevisiae* ORF YGL243w/HRA400 and mammalian ADARs was first noticed by Vandenbol *et al*. (1995). In particular, HRA400p is 22% identical and 46% similar to the C-terminal part of mammalian ADARs. Here, we show that this yeast protein is indeed an adenosine deaminase which specifically converts A to I in tRNA but not in pre-mRNA or fully duplexed RNA in contrast to mammalian ADAR1 and ADAR2. This is the first report of the cloning and characterization of a tRNA-specific adenosine deaminase.

The sequence homology between Tad1p and the mammalian RNA editing enzymes ADAR1 and ADAR2 extends through the entire ORF of *TAD1* and is not limited to the characteristic deaminase domain found in all ADAR enzymes (boxed in Figure 1B). This suggests that the two classes of enzymes, the mammalian pre-mRNA editing enzymes and the yeast tRNA modification enzyme, did not obtain catalytic capacity by independent evolution of a similar catalytic center, but more likely, the enzymes are directly related to each other. We therefore regard the yeast enzyme Tad1p as a member of the ADAR enzyme

Table I. Substrate specificity of Tad1p

tRNA/species	wt/mutations	Inosine(s) assayed	*S.cerevisiae* S100 extract mol I/mol tRNA	Tadp1 (recombinant) mol I/mol tRNA
Ala/yeast	wt	I_{34} and I_{37}	1.7 ± 0.2	0.8 ± 0.1
	A34G	I_{37}	0.6 ± 0.1	0.8 ± 0.1
	A34G and ΔU13	I_{37}	0	0
	ΔU33	I_{34} and I_{37}	0	0
Ala/*B.mori*	wt	I_{34} and I_{37}	1.3 ± 0.1	0.35 ± 0.05
	A34G	I_{37}	0.55 ± 0.05	0.4 ± 0.1
	A37G	I_{34}	0.6 ± 0.1	0
	U8C	I_{34} and I_{37}	0.9 ± 0.1	0.15 ± 0.05
	A48C	I_{34} and I_{37}	1.1 ± 0.1	0
Ala/*E.coli*	wt	I_{37}	0	0
Asp/yeast (G_1–C_{73})	GU3435AA	I_{34}	0.5 ± 0.05	0
	GU3435AA and G37A	I_{34} and I_{37}	0.2 ± 0.03	0
	G37A	(I_{37})	0	0
	AC-loop tRNA-Ala	I_{34} and I_{37}	0.8 ± 0.05	0

tRNA-specific adenosine deaminase assay. Synthetic tRNAs from different organisms and mutants were incubated with purified recombinant Tad1p (0.2 ng) or yeast S100 extracts (10 μg total protein) for 1 h at 30°C under standard assay conditions. Values were determined in at least two independent experiments.

family, although it lacks dsRBDs. The presence of dsRBDs in ADAR1 and ADAR2 was shown to be necessary for recognition of their editing substrates and the ability to convert A to I in extended dsRNA (Lai *et al*., 1995; Maas *et al*., 1996). Tad1p does not require such domains to bind to the tRNA substrate. Perhaps the yeast enzyme binds its tRNA with a yet uncharacterized RNA-binding domain or via residues localized within its deaminase domain in a manner similar to APOBEC-1 (the catalytic subunit of the apolipoprotein B mRNA editing multienzyme complex) which contains a novel low affinity RNA-binding domain (reviewed in Smith *et al*., 1997; see also Navaratnam *et al*., 1998).

We considered the possibility that in yeast the dsRBDs might be encoded by a separate gene, the product of which would complement the missing dsRBDs by forming a functional complex with the catalytic polypeptide Tad1p. However, *S.cerevisiae* and *P.pastoris* extracts containing Tad1p or pure recombinant Tad1p failed to deaminate A in extended dsRNA, the 'classical' *in vitro* substrate for ADAR1 and ADAR2, nor did they edit specific adenosines in the pre-mRNA of GluR-B. The lack of a detectable dsRNA-dependent adenosine deaminase activity in yeast extracts suggests that yeast (and possibly all lower eukaryotes) do not contain such an activity and that this may have appeared in higher eukaryotes only. Conversely, the human ADAR1 and ADAR2 enzymes do not catalyze the formation of any detectable amounts of I in $tRNA^{Ala}$ from yeast or *B.mori in vitro*. However, we cannot exclude the possibility that such a reaction might occur *in vivo*, with the aid of cofactors, even though *in vitro* the ADARs do not require any protein cofactors for activity.

Pure recombinant Tad1p/scADAT1 converts A to I at position 37 in eukaryotic $tRNA^{Ala}$ but not at at position 34 (Figure 3A). This indicates that a second tRNA-specific adenosine deaminase is required in the cell to carry out this function. Our results obtained *in vivo* support this hypothesis. RT–PCR carried out on isolated $tRNA^{Ala}$ from yeast *tad1-1* mutant cells reveals the presence of I_{34} and an unmodified A_{37} (Figure 3B). In addition, extracts prepared from the *tad1-1* mutant are catalyzing the formation of I_{34} normally, whereas the activity for A_{37} deamination is lacking (Figure 4). Thus, the two reactions must be catalyzed by different enzymes.

Enzymatic formation of I_{37} in $tRNA^{Ala}$ depends on tRNA conformation

By comparing different tRNA substrates we have demonstrated that Tad1p/scADAT1 is highly specific for yeast $tRNA^{Ala}$ (K_m = 6 ± 4 nM). Neither *E.coli* $tRNA^{Ala}$ or a chimeric yeast $tRNA^{Asp}$ bearing the anticodon stem–loop of yeast $tRNA^{Ala}$ were substrates for Tad1p (see Table I). In addition, mutant $tRNA^{Ala}$ from yeast and *B.mori* with a shortened anticodon-loop and potentially altered 3D structures were almost inactive in the enzymatic reaction catalyzed by Tad1p. We also observed a clear dependence of A_{37}–I_{37} conversion on Mg^{2+} ions in the *in vitro* reaction. This requirement may also merely reflect the necessity of maintaining a correct 3D architecture of the tRNA substrate rather than an involvement in catalysis (Uhlenbeck, 1995). One reason why the *in vitro* reaction did not go to completion under certain experimental conditions may be that a fraction of the tRNA population was not correctly folded *in vitro*. Such imperfect folding may be favored in tRNAs transcribed *in vitro* which lack the many modifications that are normally present *in vivo*. It has been reported that various tRNA modifications can influence the stability and structure of tRNAs (Björk, 1995; Steinberg and Cedergren, 1995).

In summary, the results strongly suggest that Tad1p requires both the local conformation of the anticodon loop and a correct folding of the L-shaped $tRNA^{Ala}$ substrate to recognize and deaminate A_{37}. Similar conclusions were previously drawn for the yeast tRNA:A_{34} deaminase with a using partially purified enzyme and a collection of 3D variants of yeast $tRNA^{Asp}$ (Auxilien *et al*., 1996). Therefore, because of its specific substrate requirement, it appears very unlikely that adenosines in mRNAs could be a substrate for Tad1p or the tRNA:A_{34} deaminase.

Tad1p is not essential for cell growth

The *TAD1* gene is not essential in yeast, and we did not observe a slow-growth phenotype for *TAD1*-disrupted

strains under standard growth conditions at 30°C. We demonstrated that I and m^1I, respectively, at position 37 in the $tRNA^{Ala}$ (IGC) cannot be formed in these mutant strains (Figure 3B). Therefore, this modification is not essential for yeast under optimal growth conditions. However, it is not certain whether a $tRNA^{Ala}$ (IGC) missing m^1I_{37} is still functional *in vivo*. Eleven gene copies of the $tRNA^{Ala}$ (IGC) exist in the *S.cerevisiae* genome and in addition five genes for another putative $tRNA^{Ala}$ with the anticodon UGC (Hani and Feldmann, 1998). Although the latter tRNA has not been sequencend, it might be possible that the $tRNA^{Ala}$ (UGC) could complement a non-functional $tRNA^{Ala}$ (IGC) missing m^1I at position 37. No function for m^1I_{37} in eukaryotic $tRNA^{Ala}$ (IGC) has been described yet, but interestingly, I_{34} as well as m^1I_{37} in human $tRNA^{Ala}$ are targets for specific autoantibodies present in the serum of patients with inflammatory muscle disease of the PL-12 polymyositis type (Bunn and Mathews, 1987). In analogy to the function assigned to m^1G_{37} found within several tRNAs from the prokaryote *Salmonella typhimurium*, one can speculate that m^1I_{37} (a modification similar to m^1G_{37}) might play a role in the prevention of translational frameshifts and improve the fidelity of translation (Björk *et al*., 1989; Qian and Björk, 1997).

Tad1p may represent the ancestor of pre-mRNA editing enzymes

The sequence conservation between Tad1p and the mammalian RNA editing enzymes ADAR1 and ADAR2 and the similarities in their biochemical behaviour suggest a possible evolutionary relationship between the two classes of enzymes. Besides the well characterized ADAR1 and ADAR2 enzymes and the brain-specific ADAR-like protein RED2 from rat, sequences encoding ADAR-like proteins are found in the nematode *Caenorhabditis elegans* (T20H4.4; U00037; Kim *et al*., 1994; Bass, 1997), the fruitfly *Drosophila melanogaster* (M.A.O'Connell, personal communication), and mouse (Tenr; Schuhmacher *et al*., 1995). Whereas the ADAR1 proteins contain three and ADAR2 and RED2 two dsRBDs, only one dsRBD was predicted for putative deaminases from *C.elegans* and mouse. The different numbers of dsRBDs may reflect a progressive acquisition of dsRBDs in the N-terminal region upstream of the deaminase domain during evolution. In this scenario, the ancestral gene may lack dsRBDs, which is true for the yeast gene *TAD1*. Alternatively, it is possible that Tad1p/scADAT1 has lost its dsRBDs during evolution to gain the new function of a tRNA-specific adenosine deaminase. We favor the first scenario in which the dsRBD building blocks may have fused to the N-terminus of an ancestral gene after the animal/fungal divergence ~10^9 years ago (Wolfe and Shields, 1997).

Covello and Gray (1993) have proposed a three-step model for the evolution of RNA editing activity suggesting that most forms of RNA editing evolved after the split of eukaryotes, prokaryotes and archaea, and independently of each other. In the first step, RNA editing appeared as an alteration of a pre-existing enzyme activity. In the second step, a mutation in a gene, which might be deleterious or lethal for the organism could be functionally repaired on the mRNA level by the co-evolving RNA editing activity. The mutation is rendered neutral by the editing machinery, acting as a sort of 'repair system'. In the third step, the editing activity is further propagated and increasing numbers of editing sites are fixed by genetic drift in the genome. Considering this model, Tad1p may represent a precursor of the pre-mRNA editing enzymes. A paralog derived by genome duplication of the ancient *TAD1*-like gene might have received mRNA-editing competence by acquisition of a dsRB-module and further genetic drift. Because the gene *TAD1* is not essential, it remains also plausible that the ancient *TAD1*-like gene product itself (without genome duplication) might have evolved to a pre-mRNA dependent editing enzyme, and the ancient function as a tRNA-specific adenosine deaminase was lost. Replacement of this function might have been obtained by recruitment of another enzyme that catalyzes a chemically similar reaction (Benner *et al*., 1993).

The common ancestor of the deaminase domain found in Tad1p and the other members of the ADAR family is not known. *TAD1* represents the only gene in the *S.cerevisiae* genome containing an ADAR-like deaminase domain. Homologous sequences to Tad1p are localized on the second chromosome in the genome of the fission yeast *Schizosaccharomyces pombe* (accession No. AL021748; 53% identity, 73% similarity). In contrast, no ADAR-like deaminase domain is present in the genome of *E.coli* K-12 (Blattner *et al*., 1997). These findings correlate with the observation that I at position 37 is unique in $tRNA^{Ala}$ from eukaryotes and has not been detected in prokaryotes. Therefore, we expect a different molecular organization of the deaminase domain in the $tRNA:A_{34}$ deaminase.

Materials and methods

Oligonucleotide primers

The PCR primers used for cloning the ORF YGL243w were YD1, 5′ GGACTAGTTTCGTGCCAAGGAACAAGG-3′ and YD2: 5′-GGACTAGTTTCACATCATCCGTTCTGG-3′, both encoding *Spe*I-restriction sites (underlined). YDreg1, 5′-CGTGAGAAAGAGCTACTTGTGGC-3′ anneals 248 bp upstream and YDreg3: 5′-AGTGAACAATTGCTGGATGCAGC-3′, 663 bp downstream of the ORF of YGL243w. YD3v, 5′-ATACTGGCATTACGAGGTGC-3′ and YD4v, 5′-GTCGTTAGGACTAAACCAGG-3′ were sequencing primers for *TAD1*. The reverse transcription, PCR and sequencing primers for yeast $tRNA^{Ala}$ were RTAG2, 5′-GACACGGTACCACACAACGGTGGACGAGTCCGGAATCG-3′ which is the reverse transcriptase primer antisense on yeast $tRNA^{Ala}$; PCRK3, 5′-GACACGGTACCACACAACGG-3′ a PCR primer for cDNA primed with RTAG2; PCRT2, 5′-GGGCGTGTGGCGTAGCCGG-3′ which is antisense to yeast $tRNA^{Ala}$.

Construction of tRNA genes

The yeast wt $tRNA^{Ala}$ gene with upstream T7 RNA polymerase promoter sequences, flanked by a *Hin*dIII-restriction site at the 5′-end and tandem *Eco*T22/*Bam*HI-restriction sites at the 3′ end, was prepared by stepwise hybridization and ligation of two sets of five complementary and partially overlapping synthetic oligonucleotides (17–34 mers) as described earlier for yeast $tRNA^{Asp}$ (Perret *et al*., 1990). Plasmids containing variants of the wt yeast $tRNA^{Asp}$ gene are described in Auxilien *et al*. (1996) and a chimeric yeast $tRNA^{Asp}$ bearing the anticodon stem and loop (nucleotide positions 27–44) identical to that of yeast $tRNA^{Ala}$ was also constructed by stepwise assembling of synthetic oligonucleotides. After purification by electrophoresis on a denaturing 10% polyacrylamide gel, the synthetic gene was ligated into the polylinker of the PUC118 phagemid and transformed in JM101 competent *E.coli* cells. The wt gene of *B.mori* $tRNA^{Ala}_2$ (Young *et al*., 1986) was kindly provided by Dr Karen Sprague (University of Oregon). This tRNA gene was amplified by PCR with two oligonucleotides complementary to the 15 terminal nucleotides and subcloned into pUC118. The primer complementary to the 5′-end sense strand contained an extra T7 promoter sequence and a *Hin*dIII-restriction

site at its 5′ end, while the one complementary to 3′-end antisense strand had a 5′ extra sequence corresponding to the tandem restriction sites *Bam*HI/*Mva*I. The resulting purified PCR product was subcloned into *Hin*dIII/*Bam*HI sites of pUC118. All variants of yeast and *B.mori* $tRNA^{Ala}$ were generated by site-directed mutagenesis on double-stranded DNA with the 'MORPH Mutagenesis Kit' (5 Prime→3 Prime Inc., Boulder, CO, USA) according to the manufacturer's recommendations and sequenced in both directions.

Cloning of the TAD1 gene and plasmids

All techniques used for manipulating DNA were as described (Sambrook *et al*., 1989). The ORF YGL243w was amplified from 10 ng *S.cerevisiae* genomic DNA by PCR with the primers YD1 and YD2, both containing *Spe*I-restriction sites, and cloned subsequently into these sites in the polylinker of pBluescript KS (Stratagene). Three clones (pKS-TAD1) from parallel PCRs were sequenced with a Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) on an Applied Biosystems 373A sequencer according to the manufacturer's instructions. In the same manner, PCR was performed with the primers YDreg1 and YDreg3 to amplify the ORF of YGL243w plus promoter and terminator sequences. The 2111 bp PCR fragment was subsequently cloned into linearized pCR2.1 (Invitrogen). The resulting plasmid pCR-YDr13 was further used to generate plasmids for disruption of the *TAD1* gene and for rescuing the *TAD1* disruption mutants AG3 and AG4.

Yeast strains, genetic methods and media

The ORF of *TAD1* (YGL243w) was disrupted in the diploid *S.cerevisiae* strain BMA41 (*MAT***a**/α, *ade2*/*ade2*, *leu2*/*leu2*, *ura3*/*ura3*, *trp1*Δ/*trp1*Δ, *his3*/*his3*) (Amrani *et al*., 1996) as follows. A blunt ended *Bgl*II fragment of the plasmid pGURT (Jenny *et al*., 1996) containing the *TRP1* gene, was inserted into pCR-YDr13 linearized either with *Stu*I/*Hin*cII or *Hin*cII only resulting in two constructs. In the first, the coding sequence for amino acids (aa) 48–186 of *TAD1* were replaced by the *TRP1* marker (pCR-YDr13T), in the second one the *TRP1* gene was inserted at aa 186 via the *Hin*cII restriction site (pCR-YDr13HT). pCR-YDr13T was cut with *Nsi*I/*Acc*I and pCR-YDr13HT with *Xho*I/*Acc*I respectively, and the fragments encompassing the *TRP1* gene were purified and transformed into yeast strain BMA41. Tryptophan prototrophs were selected and the correct genomic integration of the *TRP1* gene was verified by PCR. In the diploid strain AG1 (*MAT***a**/α, *ade2*/*ade2*, *leu2*/*leu2*, *ura3*/*ura3*, *trp1*Δ/*trp1*Δ, *his3*/*his3*, *TAD1*/*tad1*::*TRP1*) *TRP1* replaces aa 48–186 of one *TAD1* allele, leading to the mutant allele *tad1-1*. In strain AG2 (*MAT***a**/α, *ade2*/*ade2*, *leu2*/*leu2*, *ura3*/*ura3*, *trp1*Δ/*trp1*Δ, *his3*/*his3*, *TAD1*/*tad1*::*TRP1*) *TRP1* is inserted at coding aa 186 of one *TAD1* allele (*tad1-2*). After sporulation and tetrad dissection (Guthrie and Fink, 1991) Trp^+ haploids of strain AG1 and AG2 were selected and will be referred to as AG3 (*ade2*, *leu2*, *ura3*, *trp1*Δ, *tad1-1*) originating from AG1 and AG4 (*ade2*, *leu2*, *ura3*, *trp1*Δ, *tad1-2*) originating from AG2, respectively. An *Eco*RI fragment of pCR-YDr13 was subcloned into the vector pFL-38 (Bonneaud *et al*., 1991), generating a centromeric plasmid with *URA3* marker and *TAD1*. The resulting construct pFL38-YDr13 was transformed into AG3. Trp^+/Ura^+ haploids were selected and are further designated AG5 (*ade2*, *leu2*, *ura3*, *trp1*Δ, *tad1-1*, pFL38-YDr13). In addition, the ORF of *TAD1* was subcloned from the *P.pastoris* expression vector pSK-$FLIS_6$-Tad1 (see below) via *Eco*RI into pGALΔTrp1 [Trp1-cassette was removed from a pGAL vector (Blum *et al*., 1989)]. In the resulting plasmid pGALΔTrp1–TAD1, the *TAD1* gene is under the control of an inducible galactose promoter. Transformation into strain AG3 and selection of Trp^+/Ura^+ haploids resulted in the isolation of strain AG6 (*ade2*, *leu2*, *ura3*, *trp1*Δ, *tad1-2*, pGALΔTrp1–TAD1).

Yeast cells were cultured in YPD or synthetic medium as described (Guthrie and Fink, 1991). The cells were harvested at OD_{600} = 1–2 and extracts were prepared in liquid nitrogen as described (O'Connell *et al*., 1998). Yeast S100 extracts were prepared according to Auxilien *et al*. (1996).

Expression of Tad1p in P.pastoris and purification of the recombinant protein

The coding sequence of *TAD1* was subcloned from pKS-TAD1 into the vector pSK-$FLIS_6$ (Gerber *et al*., 1997; O'Connell *et al*., 1998) via *Spe*I restriction sites and analyzed for correct orientation. Electroporation of pSK-$FLIS_6$–Tad1 into the *P.pastoris* strain GS115 (Invitrogen), screening and expression were carried out as described (Gerber *et al*., 1997; O'Connell *et al*., 1998). Expression of the recombinant protein was monitored by immunoblot analysis with a mouse anti-FLAG M2 monoclonal antibody 1:5000 (Eastman Kodak). The cells were harvested at OD_{600} = 28 and the pellet was washed twice with water and buffer A (50 mM HEPES–KOH pH 7.9, 50 mM KCl, 10 % –glycerol, 1 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml pepstatin, 0.4 μg/ml leupeptin) and liquid nitrogen extracts were prepared.

All further manipulations were carried out at 4°C; fractions were frozen in liquid nitrogen and stored at –70°C. The lysate (100ml) was mixed with 2 ml of a 50% slurry of Ni^{2+}-nitrilotriacetic acid (NTA) agarose (Qiagen) in buffer A, adsorbed for 30 min on ice and poured into a column. The column was washed with buffer A containing 10 mM imidazole (pH 8.0) and the recombinant protein was eluted with buffer A containing 250 mM imidazole (pH 8.0). EDTA (pH 8.0) was added to a final concentration of 2.5 mM to each fraction. Aliquots from fractions were analyzed by electrophoresis on a 10% SDS–polyacrylamide gel and proteins were either stained with Coomassie Blue R-250 (Bio-Rad) or detected with the anti-FLAG antibody. Enzymatic activity was determined by a tRNA-specific adenosine deaminase assay. Peak fractions of the Ni/NTA column were loaded on a 1 ml HiTrap™ SP (Pharmacia) column that had been equilibrated in buffer B [buffer A containing 2.5 mM EDTA, 0.01% NP-40 and 1 mM dithiothreitol (DTT) instead of β-mercaptoethanol]. The column was washed with 10 column volumes buffer B and developed with a 30 ml gradient from 50 mM to 1 M KCl. 0.5 ml fractions were collected and analyzed as described above. tRNA editing activity eluted from 100–200 mM KCl. Half of the active pool of the HiTrap™ SP column was loaded on a Superdex™ 200 gel filtration column (HiLoad™ 16/60 prep grade, Pharmacia). The column was run with buffer C (buffer B containing 20% glycerol) and 1 ml fractions were collected. A peak of Tad1p activity eluted after 78 ml. The column was standardized with aldolase (158 kDa, Stokes' radius = 48.1 Å), bovine serum albumin (BSA) (63.3 kDa, 35.5 Å) and ovalbumin (42.7 kDa, 30.5 Å). Fractions 77–79 were pooled and concentrated in a Centricon 10 (Amicon) at 4°C. Aliquots were further used for *in vitro* activity assays. Protein concentrations were determined by the Bradford method (Bradford, 1976) or on a silver stained 10% SDS–polyacrylamide gel with different amounts of BSA as a reference standard.

In vitro transcription of the tRNA genes and enzyme assays

In vitro T7 RNA polymerase transcription with [α-^{33}P]ATP and purification of the transcripts on 6% urea gels was mainly performed as described (Auxilien *et al*., 1996), except that the transcription mixture was incubated with 10 units of RNase-free DNase I (Boehringer, Mannheim) for 20 min at 37°C before being loaded on the gel.

Tad1p activity was assayed by TLC of nuclease P1-digested RNA products (O'Connell and Keller, 1994; Auxilien *et al*., 1996) with minor modifications. The reaction mixture was optimized for Tad1p activity and contained 50 mM Tris–HCl (pH 7.9), 25 mM KCl, 2.5 mM $MgSO_4$, 0.1 mM EDTA (pH 8.0), 1 mM DTT, 10% glycerol and 50–200 fmol of ^{33}P-labeled tRNA substrate in a 50 μl reaction. 0.2 mg/ml BSA was added to the reaction when pure enzyme was used. To test m^1I formation, 0.02 mM SAM was added to the reaction mixture. Reactions were incubated for 1 h at 30°C. The RNA was ethanol-precipitated, washed with 70% ethanol and vacuum dried. The pellets were resuspended in 10 μl of P1 buffer (30 mM KOAc pH 5.3, 10 mM $ZnSO_4$) and digested with 0.5 μg of P1 nuclease (Boehringer, Mannheim) overnight at 37°C. Unlabelled 5′ IMP (Sigma) was added to the reaction mixture as an internal standard. Reaction products were separated by one or two-dimensional TLC and quantified on a PhosphorImager 425 (Molecular Dynamics) as described (Auxilien *et al*., 1996; Grosjean *et al*., 1996a).

K_m and k_{cat} were determined on yeast wt $tRNA^{Ala}$ (IGC). The substrate was titrated in the concentration range from 0.5–15 nM and incubated with pure recombinant Tad1p. The reaction was performed in 50 μl for 15–30 min at 30°C and products were digested and quantified as described above. K_m and V_{max} were determined graphically from a Lineweaver–Burk plot. The average of three independent K_m and V_{max} determinations (performed in duplicate) corresponds to the indicated values.

Sequence analysis of edited tRNAs

RT–PCR amplification and subsequent sequencing was performed with *in vitro* edited tRNAs and with total RNA isolated from yeast strains. Total RNA was isolated from yeast cultures with the 'Total RNA Midi Kit' (Qiagen) according to the manufacturer's instructions. Precipitated RNA was eluted in sterile water and 50 fmol were heated with 5μM RTAG2 primer in a volume of 20 μl for 10 min at 70°C. After cooling on ice 10 μl of reverse transcription mix [6 μl 5×reverse transcription buffer, 1.5 μl 0.1 M DTT, 1.5 μl 10 mM each of dATP, dCTP, dGTP, dTTP (dNTPs), 1 μl RNAguard RNase inhibitor (Pharmacia)] was added to the reaction and mixed. 10 μl of the mix were removed for mock incubation without reverse transcriptase. One μl (5U) of reverse

transcriptase (Superscript™ II RT, Life Technologies, Inc.) was added to the other 20 µl of the mix and the reaction was incubated for 1 h at 42°C and 5 min at 95°C. PCR was carried out with 2 µl of the RT reaction and the mock reaction respectively. The 50 µl PCR contained 1× PCR buffer (10 mM Tris–HCl pH 8.5, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs), and 0.3 µM of each oligonucleotide primer PCRT2 and PCRK3. The reaction mixture was heated to 95°C for 3 min and after cooling to 80°C, 2.5 U of *Taq*-polymerase (Boehringer) were added to each sample and 25 cycles were completed (30 s at 94°C; 30 s at 56°C; 45 s at 72°C). The PCR products were run on a 2.5% agarose gel and purified. Approximately 50 ng was used in a sequencing reaction with Dye Terminators (Perkin Elmer) and supplied to an automated sequencer as described above. Alternatively, the gel-purified fragments were subcloned into linearized pCR2.1 (Invitrogen) and individual clones were sequenced with M13 reverse primer (Invitrogen).

Acknowledgements

We are grateful to Drs Peter H.Seeburg (Max-Planck-Institut für medizinische Forschung, Heidelberg, Germany) and Mary A.O'Connell (MRC Human Genetics Unit, Edinburgh, UK) for their generous help initiating this project. We thank Qiang Yu for construction of the synthetic yeast $tRNA^{Ala}$ gene, Colette Simon, Yves Corda and Sylvie Auxilien (Gif-sur-Yvette, France) for the preparation of the mutants of yeast and *B.mori* $tRNA^{Ala}$ and of yeast $tRNA^{Asp}$ and Dr Micheline Vandenbol (Agronomy Faculty of Gembloux, Belgium) for providing the cosmid EGH 175 carrying the HRA400 gene. We thank Ursula Rüegsegger, Mary A.O'Connell, Lionel Minvielle-Sebastia, Martin Ohnacker and Elmar Wahle for critically reading the manuscript. The work in Basel was supported by the Kantons of Basel, the Swiss National Science Foundation and the Human Frontiers Science Program. A.G. is a recipient of a graduate student fellowship from the Boehringer Ingelheim Fonds. Work in Gif-sur-Yvette was supported by a grant to H.G. from the CNRS and from the 'Association pour la Recherche sur le Cancer'.

References

Amrani,N., Dufour,M.-E., Bonneaud,N. and Lacroute,F. (1996) Mutations in *STS1* suppress the defect in 3′ mRNA processing caused by the *rna15–2* mutation in *S.cerevisiae*. *Mol. Gen. Genet.*, **252**, 552–562.

Auxilien,S., Crain,P.F., Trewyn,R.W. and Grosjean,H. (1996) Mechanism, specificity and general properties of the yeast enzyme catalysing the formation of inosine 34 in the anticodon of transfer RNA. *J. Mol. Biol.*, **262**, 437–458.

Basilio,C., Wahba,A.J., Lengyel,P., Speyer,J.F. and Ochoa,S. (1962) Synthetic polynucleotides and the amino acid code. *Proc. Natl Acad. Sci. USA*, **48**, 613–616.

Bass,B.L. (1997) RNA editing and hypermutation by adenosine deamination. *Trends Biochem. Sci.*, **22**, 157–162.

Bass,B.L., Nishikura,K., Keller,W., Seeburg,P.H., Emeson,R.B., O'Connell,M.A., Samuel,C.E. and Herbert,A. (1997) A standardized nomenclature for adenosine deaminases that act on RNA. *RNA*, **3**, 947–949.

Benner,S.A., Cohen,M.A., Gonnet,G.H., Berkowitz,D.B. and Johnsson,K.P. (1993) Reading the palimpsest: contemporary biochemical data and the RNA world. In Gesteland,R.F. and Atkins,J.F. (eds), *The RNA World*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 27–70.

Björk,G.R. (1995) Biosynthesis and function of modified nucleosides. In Söll,D. and RajBhandary,U.L. (eds), *tRNA: Structure, Biosynthesis and Function*. American Society Microbiology Press, Washington DC, pp. 165–205.

Björk,G.R., Wikström,P.M. and Byström,A.S. (1989) Prevention of translational frameshifting by the modified nucleoside 1-methylguanosine. *Science*, **244**, 986–990.

Blattner,F.R. *et al.* (1997) The complete genome sequence of *Escherichia coli* K-12. *Science*, **277**, 1453–1474.

Blum,S., Mueller,M., Schmid,S.R., Linder,P. and Trachsel,H. (1989) Translation in *Saccharomyces cerevisiae*: initiation factor eIF-4A-dependent cell-free system. *Proc. Natl Acad. Sci. USA*, **86**, 6043–6046.

Bonneaud,N., Ozier-Kalogeropoulos,O., Li,G., Labouesse,M., Minvielle-Sebastia,L. and Lacroute,F. (1991) A family of low and high copy replicative, integrative and single-stranded *S. cerevisiae*/*E.coli* shuttle vectors. *Yeast*, **7**, 609–615.

Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

Bunn,C.C. and Mathews,M.B. (1987) Autoreactive epitope defined as the anticodon region of alanine tRNA. *Science*, **238**, 1116–1119.

Burns,C.M., Chu,H., Rueter,S.M., Hutchinson,L.K., Canton,H., Sanders-Bush,E. and Emeson,R.B. (1997) Regulation of serotonin-2C receptor G-protein coupling by RNA editing. *Nature*, **387**, 303–308.

Carter,C.W. (1995) The nucleoside deaminases for cytidine and adenosine: structure, transition state stabilization, mechanism and evolution. *Biochimie*, **77**, 92–98.

Covello,P.S. and Gray,M.W. (1993) On the evolution of RNA editing. *Trends Genet.*, **9**, 265–268.

Dabiri,G.A., Lai,F., Drakas,R.A. and Nishikura,K. (1996) Editing of the GluR-B ion channel RNA *in vitro* by recombinant double-stranded RNA adenosine deaminase. *EMBO J.*, **15**, 34–45.

Devereux,J., Haeberli,P. and Smithies,O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.*, **12**, 387–395.

Dirheimer,G., Keith,G., Dumas,P. and Westhof,E. (1995) Primary, secondary and tertiary structures of tRNAs. In Söll,D. and RajBhandary,U.L. (eds), *tRNA: Structure, Biosynthesis and Function*. American Society of Microbiology, Washington, DC, pp. 93–126.

Gerber,A., O'Connell,M.A. and Keller,W. (1997) Two forms of human double-stranded RNA-specific editase 1 (hRED1) generated by the insertion of an Alu cassette. *RNA*, **3**, 453–463.

Grosjean,H. *et al*. (1996a) Enzymatic formation of adenosine to inosine and to N^1-methylinosine in transfer RNAs: A review. *Biochimie*, **78**, 488–501.

Grosjean,H., Edqvist,J., Stråby,K.B. and Giegé,R. (1996b) Enzymatic formation of modified nucleosides in tRNA: dependence on tRNA structure. *J. Mol. Biol.*, **255**, 67–85.

Guthrie,C. and Fink,G.R. (1991) *Guide to Yeast Genetics and Molecular Biology*. Academic Press, San Diego, CA.

Hani,J. and Feldmann,H. (1998) tRNA genes and retroelements in the yeast genome. *Nucleic Acids Res.*, **26**, 689–696.

Higuchi,M., Single,F.N., Köhler,M., Sommer,B., Sprengel,R. and Seeburg,P.H. (1993) RNA editing of AMPA receptor subunit GluR-B: A base-paired intron–exon structure determines position and efficiency. *Cell*, **75**, 1361–1370.

Holley,R.W., Everett,G.A., Madison,J.T. and Zamir,A. (1965) Nucleotide sequences in yeast alanine transfer RNA. *J. Biol. Chem.*, **240**, 2122–2127.

Hough,R.F. and Bass,B.L. (1997) Analysis of *Xenopus* dsRNA adenosine deaminase cDNAs reveals similarities to DNA methlytransferases. *RNA*, **3**, 356–370.

Jenny,A., Minvielle-Sebastia,L., Preker,P.J. and Keller,W. (1996) Sequence similarity between the 73 kDa protein of mammalian CPSF and a subunit of yeast polyadenylation factor I. *Science*, **274**, 1514–1517.

Kable,M.L., Heidmann,S. and Stuart,K.D. (1997) RNA editing: getting U into RNA. *Trends Biochem. Sci.*, **22**, 162–166.

Kim,U., Wang,Y., Sanford,T., Zeng,Y. and Nishikura,K. (1994) Molecular cloning of cDNAs for double-stranded RNA adenosine deaminase, a candidate enzyme for nuclear RNA editing. *Proc. Natl Acad. Sci. USA*, **91**, 11457–11461.

Kröger,M. and Singer,B. (1979) Ambiguity and transcriptional errors as a result of methylation of N-1 purines and N-3 of pyrimidines. *Biochemistry*, **18**, 3493–3500.

Lai,F., Drakas,R. and Nishikura,K. (1995) Mutagenic analysis of double-stranded RNA adenosine deaminase, a candidate enzyme for RNA editing of glutamate-gated ion channel transcripts. *J. Biol. Chem.*, **270**, 17098–17105.

Lai,F., Chen,C.-X., Carter,K.C. and Nishikura,K. (1997) Editing of glutamate receptor B subunit ion channel RNAs by four alternatively spliced DRADA2 double-stranded RNA adenosine deaminases. *Mol. Cell. Biol.*, **17**, 2413–2424.

Liu,Y., George,C.X., Patterson,J.B. and Samuel,C. (1997) Functionally distinct double-stranded RNA-binding domains associated with alternative splice site variants of the interferon-inducible double-stranded RNA-specific adenosine deaminase. *J. Biol. Chem.*, **272**, 4419–4428.

Maas,S., Melcher,T., Herb,A., Seeburg,P.H., Keller,W., Krause,S., Higuchi,M. and O'Connell,M.A. (1996) Structural requirements for RNA editing in glutamate receptor pre-mRNA by recombinant double-stranded RNA adenosine deaminase. *J. Biol. Chem.*, **271**, 12221–12226.

Maas,S., Melcher,T. and Seeburg,P.H. (1997) Mammalian RNA-dependent deaminases and edited mRNAs. *Curr. Opin. Cell Biol.*, **9**, 343–349.

Melcher,T., Maas,S., Higuchi,M., Keller,W. and Seeburg,P.H. (1995) Editing of AMPA receptor GluR-B pre-mRNA *in vitro* reveals site-selective adenosine to inosine conversion. *J. Biol. Chem.*, **270**, 8566–8570.

Melcher,T., Maas,S., Herb,A., Sprengel,R., Higuchi,M. and Seeburg,P.H. (1996a) RED2, a brain specific member of the RNA-specific adenosine deaminase family. *J. Biol. Chem.*, **271**, 31795–31798.

Melcher,T., Maas,S., Herb,A., Sprengel,R., Seeburg,P.H. and Higuchi,M. (1996b) A mammalian RNA editing enzyme. *Nature*, **379**, 460–464.

Mittaz,L., Scott,H.S., Rossier,C., Seeburg,P.H., Higuchi,M. and Antonarakis,S.E. (1997) Cloning of a human RNA editing deaminase (ADARB1) of glutamate receptors that maps to chromosome 21q22.3. *Genomics*, **41**, 210–217.

Navaratnam,N. *et al.* (1998) *Echerichia coli* cytidine deaminase provides a molecular model for apoB RNA editing and a mechanism for RNA substrate recognition. *J. Mol. Biol.*, **275**, 695–714.

O'Connell,M.A. (1997) RNA editing: rewriting receptors. *Curr. Biol.*, **7**, 437–439.

O'Connell,M.A. and Keller,W. (1994) Purification and properties of double-stranded RNA-specific adenosine deaminase from calf thymus. *Proc. Natl Acad. Sci. USA*, **91**, 10596–10600.

O'Connell,M.A., Krause,S., Higuchi,M., Hsuan,J.J., Totty,N.F., Jenny,A. and Keller,W. (1995) Cloning of cDNAs encoding mammalian double-stranded RNA-specific adenosine deaminase. *Mol. Cell. Biol.*, **15**, 1389–1397.

O'Connell,M.A., Gerber,A. and Keller,W. (1997) Purification of human double-stranded RNA-specific editase 1 (hRed1) involved in editing of brain glutamate receptor B pre-mRNA. *J. Biol. Chem.*, **272**, 473–478.

O'Connell,M.A., Gerber,A. and Keegan,L.P. (1998) Purification of native and overexpression of recombinant dsRNA-specific adenosine deaminases. *Methods Companion, Methods Enzymol.*, **15**, 51–62.

Paul,M.S. and Bass,B.L. (1998) Inosine exists in mRNA at tissue-specific levels and is most abundant in brain mRNA. *EMBO J.*, **17**, 1120–1127.

Perret,V., Garcia,A., Puglisi,J.D., Grosjean,H., Ebel,J.-P., Florentz,C. and Giegé,R. (1990) Conformation in solution of yeast $tRNA^{Asp}$ transcripts deprived of modified nucleotides. *Biochimie*, **72**, 735–744.

Polson,A.G., Bass,B.L. and Casey,J.L. (1996) RNA editing of hepatitis delta virus antigenome by dsRNA-adenosine deaminase. *Nature*, **380**, 454–456.

Polson,A.G., Ley III,H.L., Bass,B.L. and Casey,J.L. (1998) Hepatitis delta virus RNA editing is highly specific for the amber/W site and is suppressed by hepatitis delta antigen. *Mol. Cell. Biol.*, **18**, 1919–1926.

Qian,Q. and Björk,G.R. (1997) Structural alterations far from the anticodon of the $tRNA^{Pro}$ of *Salmonella typhimurium* induce +1 frameshifting at the peptidyl-site. *J. Mol. Biol.*, **273**, 978–992.

Rueter,S.M. and Emeson,R.B. (1998) Adenosine-to-inosine conversion in mRNA. In Grosjean,H. and Benne,R. (eds), *Modification and Editing of RNA*. American Society Microbiology Press, Washington DC, pp. 343–361.

Sambrook,S., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schuhmacher,J.M., Lee,K., Edelhoff,S. and Braun,R.E. (1995) Distribution of Tenr, an RNA-binding protein, in a lattice-like network within the spermatid nucleus in the mouse. *Biol. Reprod.*, **52**, 1274–1283.

Seeburg,P.H. (1996) The role of RNA editing in controlling glutamate receptor channel properties. *J. Neurochem.*, **66**, 1–5.

Silberklang,M., Gillum,A.M. and RajBhandary,U.L. (1979) Use of *in vitro* ^{32}P labeling in the sequence analysis of nonradioactive tRNAs. *Methods Enzymol.*, **50**, 58–109.

Smith,H.C., Gott,J.M. and Hanson,M.R. (1997) A guide to RNA editing. *RNA*, **3**, 1105–1123.

Sprinzl,M., Horn,C., Brown,M., Ioudovitch,A. and Steinberg,S. (1998) Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res.*, **26**, 148–153.

St Johnston,D., Brown,N.H., Gall,J.G. and Jantsch,M. (1992) A conserved double-stranded RNA-binding domain. *Proc. Natl Acad. Sci. USA*, **89**, 10979–10983.

Steinberg,S. and Cedergren,R. (1995) A correlation between N^2-dimethylguanosine presence and alternate tRNA conformers. *RNA*, **1**, 886–891.

Uhlenbeck,O.C. (1995) Keeping RNA happy. *RNA*, **1**, 4–6.

Vandenbol,M., Durand,P., Portetelle,D. and Hilger,F. (1995) The sequence of an 11.1 kb DNA fragment between ADH4 and ADE5 on the left arm of chromosome VII reveals the presence of eight open reading frames. *Yeast*, **11**, 1519–1523.

Wolfe,K.H. and Shields,D.C. (1997) Molecular evidence for an ancient duplication of the entire yeast genome. *Nature*, **387**, 708–713.

Young,L.S., Takahashi,N. and Sprague,K.U. (1986) Upstream sequences confer distinctive transcriptional properties on genes encoding silkgland-specific $tRNA^{Ala}$. *Proc. Natl Acad. Sci. USA*, **83**, 374–378.

Received May 6, 1998; revised and accepted June 17, 1998