

Transfer RNA binding protein in the nucleus of *Saccharomyces cerevisiae*

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Abstract

A yeast nuclear protein that binds to tRNA was identified using a RNA mobility shift assay. Northwestern blotting and N-terminal sequencing experiments indicate that this tRNA-binding protein is identical to zuotin which has previously been shown to bind to Z-DNA [(1992) EMBO J. 11, 3787–3796]. Labeled tRNA and poly(dG-m⁵dC) stabilized in the Z-DNA form identify the same protein on a Northwestern blot. In a gel retardation assay poly(dG-m⁵dC) in the Z-form strongly diminishes the binding of tRNA to zuotin. These studies establish that zuotin is able to bind to both tRNA and Z-DNA. Zuotin may be transiently associated with tRNA in the nucleus of yeast cells and play a role in its processing or transport to the cytoplasm.

Key words: tRNA; *Saccharomyces cerevisiae*; Zuotin; Nuclear protein

1. Introduction

In eukaryotic cells the primary transcripts of tRNAs contain 5' extended leader sequences, 3' trailer sequences and intervening sequences (for tRNA precursors derived from intron containing genes) and must be processed post-transcriptionally to yield functional gene products. This includes removal of the 5' and 3' additional sequences, splicing, addition of the 3'-terminal CCA sequence and modification of some bases. Most of the events involved in the maturation of precursor tRNAs occur in the nucleus before the tRNAs are exported to the cytoplasm. Because tRNA transport from the nucleus to the cytoplasm resembles a carrier-mediated process rather than simple diffusion through the nuclear pores [1,2] it is likely that further interactions with structural proteins of the nucleus are necessary for this process.

We have been interested in identifying tRNA binding proteins in the nucleus of the yeast *Saccharomyces cerevisiae*. A RNA mobility shift assay was used to detect and identify tRNA binding proteins in yeast extracts. Here we report the partial purification of a nuclear tRNA binding protein from the yeast *Saccharomyces cerevisiae*. Unexpectedly N-terminal sequencing of this protein reveals that it is the previously identified nuclear protein zuotin, a putative Z-DNA binding protein [3]. The exact biological function of zuotin is not known but the experiments reported here suggest that it could be involved in processing or transport of tRNA to the cytoplasm.

2. Materials and methods

2.1. Yeast strain and media

The genotype of the yeast *Saccharomyces cerevisiae* used in this work is as follows: AB1380 (MAT α , Ψ^+ , ura 3, trp 1, ade 2-1, can 1-100, lys 2-1, his 5). Cells were grown in YPD medium (1% yeast extract, 2% bactopectone and 2% glucose) supplemented with 10 μ g/ml adenine at 27°C.

2.2. Purification of yeast proteins

Crude total cell extract was prepared from mid-log phase yeast cells. Yeast cells from 3 l cultures were collected at 1500 \times g for 10 min at 4°C and washed twice with ice-cold water. The cell pellet was resuspended in 50 ml of zymolyase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 M sorbitol) containing 30 mM DTT and incubated for 15 min at room temperature. Cells were collected by centrifugation, resuspended in 30 ml of zymolyase buffer containing 1 mM DTT and incubated with zymolyase 100T at 30°C for 30 min to 50 min until spheroplasting was complete. The spheroplasts were centrifuged at 1500 \times g for 5 min and washed three times in ice-cold zymolyase buffer. The spheroplasts were then resuspended in 20 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 10 mM sodium acetate, 6 mM 2-mercaptoethanol and 1 mM PMSF) and lysed with 15 strokes of a tight-fitting pestle in a Dounce homogenizer. One volume of extraction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 10 mM sodium acetate, 0.8 M ammonium sulfate, 20% glycerol, 6 mM 2-mercaptoethanol and 1 mM PMSF) was added to the lysed spheroplasts and the suspension was centrifuged for 90 min at 1500 \times g. The supernatant was dialysed against storage buffer (20 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 20% glycerol, 50 mM KCl, 6 mM 2-mercaptoethanol and 1 mM PMSF) and loaded onto a 40 ml DEAE-Sephacel (Pharmacia/LKB) column equilibrated in buffer A. The column was washed with 100 ml of buffer A. After washing, a 200 ml linear gradient from 50 mM KCl to 500 mM KCl in buffer A was applied. Fractions were dialysed against buffer A and assayed for tRNA binding. The active fractions were loaded onto a 10 ml Heparin-Ultrogel A4R (IBF) column. The column was washed with 40 ml of buffer A followed by a 40 ml linear gradient from 50 mM KCl to 500 mM KCl in buffer A. Fractions containing the tRNA binding activity were dialysed against buffer A, concentrated on a Centricon microconcentrator (Amicon) and loaded onto a Hydroxyapatite-HPLC column (TSK HA, Supelco). The column was washed with 10 mM potassium phosphate buffer, pH 6.5, containing 0.01 mM CaCl₂, and a 50 ml linear gradient from 10 mM potassium phosphate buffer to 500 mM potassium phosphate buffer was applied. One ml fractions were collected, dialysed against buffer A and assayed for tRNA binding.

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2.3. Gel retardation assay

Crude nuclear extract or fractionated proteins were incubated with 10 ng of labeled tRNA in 20 μ l of 1 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 25 mM KCl, 6 mM 2-mercaptoethanol, 1 mM PMSF and 10% glycerol.

To reduce nonspecific binding, an excess (0.25 μ g/ μ l) of unlabeled poly(rA)RNA was included in the reaction mixture. After incubation for 15 min on ice the reactions were loaded on vertical (16 \times 25 \times 0.12 cm) 6% native polyacrylamide gels (acrylamide:bisacrylamide 39:1 w/w) in 0.25 \times TBE buffer which had been prerun at 180 V for 30 min at room temperature. Electrophoresis was carried out at room temperature at 180 V for 2–3 h. Gels were dried onto 3 MM Whatman paper and subjected to autoradiography.

2.4. Protein electrophoresis and Northwestern blotting

One-dimensional electrophoresis of proteins was performed on a 10% polyacrylamide-SDS gel as described by Laemmli [4]. Two-dimensional nonequilibrium pH gradient gel electrophoresis was performed as described by O'Farrell et al. [5] with an ampholine gradient of pH 3 to 10 in the first dimension and a polyacrylamide-SDS gel in the second dimension.

For Northwestern blotting, proteins were transferred overnight on a nitrocellulose membrane (BAS 85, Schleicher and Schuell) in the cold room at 20 V in a running buffer containing 20% ethanol, 0.2 M glycine and 25 mM Tris. After transfer, the membrane was incubated in a renaturation buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM MgCl₂, Denhardt solution 1 \times for three times one hour. After renaturation the membrane was incubated in the renaturation buffer containing the labeled probe and an excess of unlabeled poly(rA)RNA to reduce nonspecific binding. To stabilize poly(dG-m5 dC) (Pharmacia) in the left handed Z-form, 10 mM MgCl₂ was added to the renaturation buffer. After one hour incubation at room temperature with the radioactive probe, the membrane was washed three times (5–10 min

each) with 10 mM Tris-HCl, pH 7.5, 50 mM NaCl. The membrane was air-dried and exposed to an X-ray film.

2.5. Sequencing of protein

For protein sequence analysis, the active Hydroxyapatite-HPLC fraction was resolved on a 10% polyacrylamide-SDS gel. After electrophoresis, the protein was electroblotted onto a ProBlott membrane (Applied Biosystems), stained for 1 min with Coomassie blue (0.1% Coomassie blue, 1% acetic acid, 40% methanol) and destained in 50% methanol. The band with an apparent molecular weight of 60 kDa was excised and N-terminally sequenced by automated Edman's degradation using an Applied Biosystems 470A protein sequencer equipped with a PTH 120 Analyser [6].

3. Results and discussion

3.1. Detection of a tRNA binding protein in nuclear extracts of the yeast *Saccharomyces cerevisiae*

To identify nuclear proteins of the yeast *Saccharomyces cerevisiae* that interact with tRNA, we prepared nuclear extracts of yeast according to the method described by Verdier et al. [7]. The interaction of proteins and tRNA was detected by an RNA mobility shift assay. In this assay, 5' end labeled tRNA was incubated with the proteins and the protein–nucleic acid interaction was detected by a reduction of the mobility of the tRNA in native polyacrylamide gels. As shown in Fig. 1A a dis-

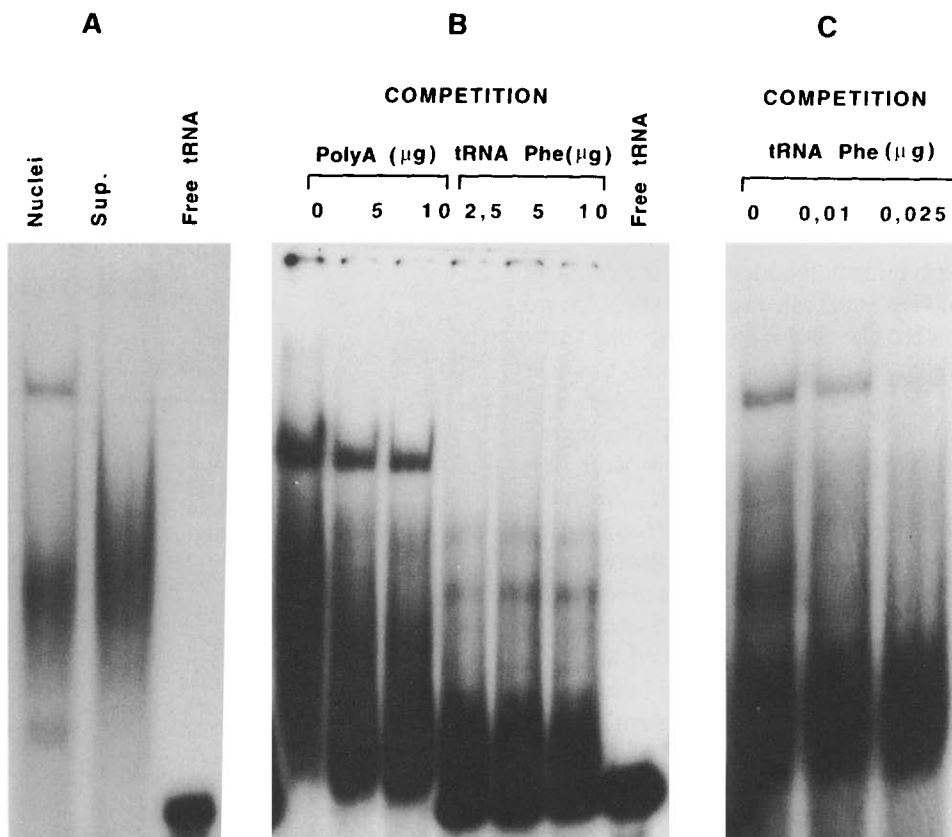


Fig. 1. Specific binding of yeast nuclear proteins to tRNA. Complex formation was monitored by non-denaturing gel electrophoresis of the 5' end labeled tRNA-protein complex. (A) Labeled tRNA^{Asp} was incubated with crude nuclear proteins (lane 1), the post-nuclear supernatant (lane 2) or without proteins (lane 3). (B and C) Labeled tRNA^{Asp} was incubated with crude nuclear proteins and increasing amounts of unlabeled competitor (poly(rA)RNA or tRNA^{Phe}).

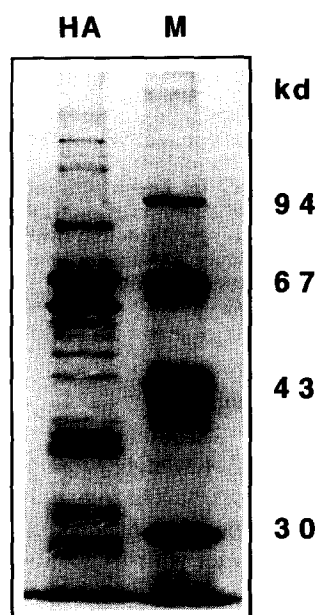


Fig. 2. Protein composition of the pooled active fractions from the Hydroxyapatite-HPLC column (HA). The proteins were resolved on a 10% polyacrylamide-SDS gel and silver stained. The molecular mass of protein markers (M) are indicated.

tinctive band-shift is formed with the proteins extracted from the yeast nuclear pellet whereas only heterogeneous complexes are formed with the proteins of the post-nuclear supernatant. The same distinctive retarded band is formed (data not shown) with several tRNAs including yeast tRNA^{Asp}, tRNA^{Phe} and initiator tRNA^{Met} or in vitro transcribed yeast tRNA^{Asp} and human initiator tRNA^{Met}.

In order to determine if the band shift is the result of specific tRNA binding, unlabeled poly(rA)RNA and several unlabeled tRNAs were used as competitors: the retarded band disappeared in the presence of a five-fold excess of unlabeled tRNA. In the experiment shown in Fig. 1B and C the labeled yeast tRNA^{Asp} was challenged with yeast tRNA^{Phe}, but other tRNA species were also able to compete with the labeled tRNA in agreement with the observation mentioned above that several tRNAs give the same retarded band on a native polyacrylamide gel. In contrast to tRNA the signal was only slightly diminished by an addition of an excess of poly(rA)RNA (more than 50% of the retarded band remained in the presence of a 2000-fold excess of poly(rA)RNA-lane 10 μ g in Fig. 1B). The formation of the heterogeneous complexes was somewhat inhibited by poly(rA)RNA (Fig. 1C). In subsequent binding assays 0.25 μ g/ μ l poly(rA)RNA was therefore included in the binding reactions.

These results suggest that the distinctive band-shift observed in the crude yeast nuclear extract reflects the formation of a specific tRNA/protein complex and that

the protein does not recognize one particular tRNA species but structural features common to all tRNAs.

3.2. Fractionation of the tRNA binding proteins

The distinctive band shift observed in nuclear extracts was also produced in whole yeast cell extracts. We therefore decided to fractionate the tRNA binding activity from whole yeast cell extracts using a combination of DEAE Sephacel, Heparin-Ultrogel and Hydroxyapatite-HPLC columns. Fractions from the DEAE Sephacel column were assayed using labeled tRNA in the presence of an excess of poly(rA)RNA. Several fractions showed positive retarded complexes. The band shift obtained with fractions eluted at about 0.25 M KCl was similar to the band shift observed in nuclear extracts. The tRNA-binding activity from the DEAE column was further purified using a Heparin-Ultrogel column followed by a Hydroxyapatite-HPLC column. The active fraction eluted from the Hydroxyapatite-HPLC column was still quite complex and included two prominent proteins with apparent molecular weights of 68 kDa and 60 kDa (Fig. 2) when analysed on a SDS-polyacrylamide gel. The band shift resulting from the interaction of this fraction with tRNA is shown in Fig. 3. A competition experiment was performed with unlabeled tRNA and unlabeled 5S RNA (Fig. 3): the retarded band produced by labeled tRNA almost completely disappeared in the presence of a 5-fold excess of unlabeled tRNA. In the presence of a 5–6-fold excess of 5S RNA more than 80% of the retarded band remained. Thus the partially purified active fraction from the HPLC column contains proteins that bind specifically to tRNA. These proteins were further characterized by Northwestern blotting.

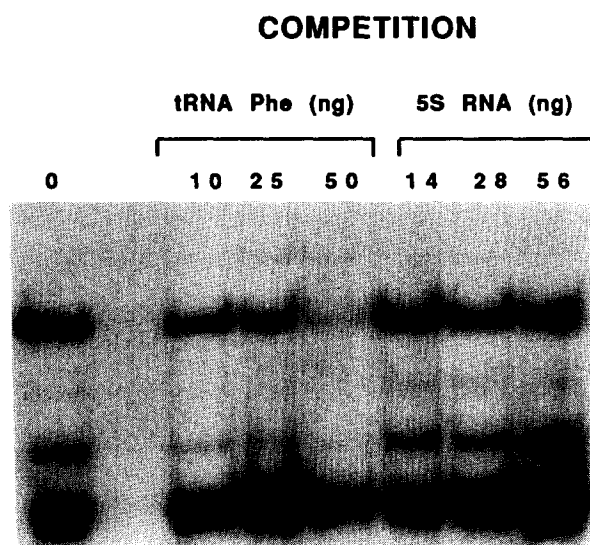


Fig. 3. Binding of the Hydroxyapatite-HPLC protein fraction to tRNA. Complex formation was monitored by non-denaturing gel electrophoresis. ³²P-labeled tRNA was incubated with the proteins in the presence of an excess of poly(rA)RNA and competitor tRNA (10 ng, 25 ng, 50 ng) or 5S RNA (14 ng, 20 ng, 56 ng).

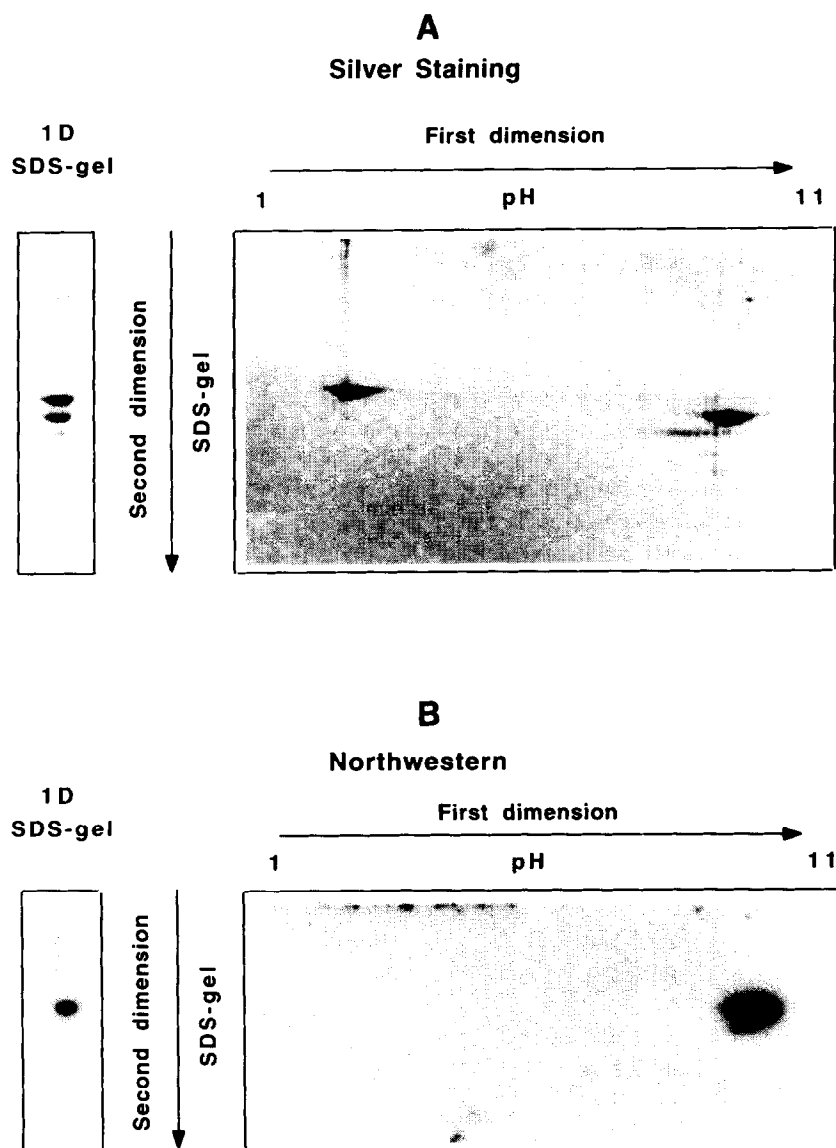


Fig. 4. Analysis of the Hydroxyapatite-HPLC protein fraction by two-dimensional gel electrophoresis. (A) Proteins were visualized by silver staining. (B) Proteins were resolved by two-dimensional gel electrophoresis and transferred to a nitrocellulose membrane. The blot was incubated with ^{32}P -labeled tRNA, washed, air-dried and exposed to an X-ray film.

3.3. Identification of the tRNA binding protein by Northwestern blotting

In order to identify the protein that interacts with the tRNA, a Northwestern blot was employed. Since the two prominent proteins in the Hydroxyapatite-HPLC fraction are very close on a SDS-polyacrylamide gel, a two-dimensional gel with a pH gradient in the first dimension and a SDS gel in the second dimension was used to separate the proteins. The 68 kDa and 60 kDa proteins have very different pIs and are well separated on the pH gradient (Fig. 4A). The measured pIs are 6.2 and 8.0 for the 68 kDa and the 60 kDa proteins, respectively. The proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane. The filter was incubated in a buffer that favors renaturation of the proteins and

subsequently incubated with ^{32}P -labeled tRNA in the same buffer. Under these conditions only one polypeptide corresponding to the prominent 60 kDa protein was detected by autoradiography (Fig. 4B).

The 60 kDa protein was gel purified for N-terminal sequencing and yielded the following sequence: MFSLPTLTSDIT. A database search revealed that this peptide sequence is present at the NH_2 -terminus of zuotin, a putative Z-DNA binding protein purified from a yeast nuclear extract [3].

In order to determine if the 60 kDa protein could bind both to Z-DNA and to tRNA, Northwestern blots were made with 5' end labeled tRNA and labeled poly(dG-m⁵dC) stabilized in the Z-form in the presence of 10 mM MgCl_2 . The two probes bound the same polypeptide

(Fig. 5) and in both cases only one protein was detected by autoradiography. To confirm that Z-DNA and tRNA bind to the same protein, Z-DNA was used as competitor in a gel retardation competition assay (Fig. 6): labeled tRNA was incubated with the protein fraction in the presence of unlabeled poly(dGm⁵dC) stabilized in the Z-form in a buffer containing 10 mM MgCl₂. As shown in Fig. 6 the shifted band bound to the labeled tRNA is strongly diminished by addition of a 6-fold molar excess of unlabeled Z-DNA indicating that tRNA and Z-DNA compete for the same protein.

4. Conclusion

In order to identify nuclear proteins that interact with tRNA we have used a gel retardation assay to purify the activities that bind to tRNA in yeast nuclear extracts. N-Terminal amino acid sequencing of a nuclear protein that recognizes tRNA reveals that it is identical to zuotin which has previously been characterized as a putative Z-DNA binding protein. Zuotin is relatively abundant in the nucleus of yeast but its function appears not to be essential since disruption of the gene ZUO1 in yeast

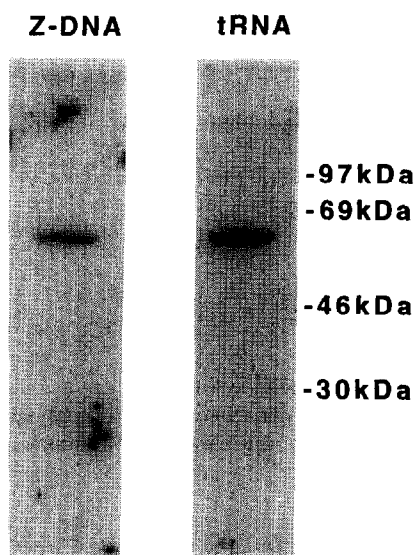


Fig. 5. Northwestern blot analysis of the Hydroxyapatite-HPLC protein fraction. The proteins were resolved on a polyacrylamide-SDS gel and transferred on a nitrocellulose filter. Prestained SDS-PAGE standards were run on the same gel and transferred to the filter. Z-DNA (the filter was incubated with ³²P-labeled poly(dG-m⁵dC) in the presence of 10 mM MgCl₂). tRNA (the filter was incubated with ³²P-labeled tRNA in the presence on an excess of poly(rA)RNA).

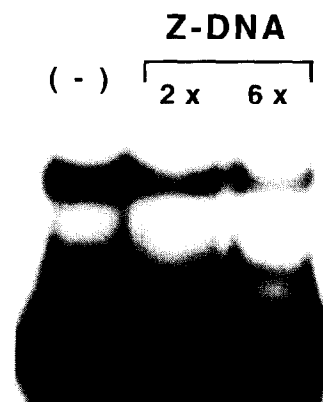


Fig. 6. Gel retardation competition assay of tRNA binding. ³²P-labeled tRNA was incubated in the presence of an excess of poly(rA)RNA and 10 mM MgCl₂ with unlabeled poly(dG-m⁵dC) (Z-DNA) at 2- or 6-fold molar excess as indicated.

results in a slow growth phenotype [3]. Zhong et al. [3] have suggested that it may be involved in some activity that is needed to maintain rapid cell growth. A role in tRNA processing or transport would be compatible with the slow growth phenotype of the disrupted mutant. It is clear, however, that the actual biological role of zuotin is not known. Since it is relatively abundant in the yeast nuclei we wonder whether it could play a role in several biological processes, possibly via interaction with nucleic acids. What is the function of the tRNA–zuotin interaction in vivo remains to be determined by using genetic and biochemical approaches.

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