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Zuotin, a DnaJ molecular chaperone, stimulates cap-independent translation in yeast

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Abstract

A small inhibitor RNA (IRNA) isolated from yeast has previously been shown to efficiently block poliovirus and hepatitis C virus IRES-mediated translation by sequestering mammalian RNA binding (transacting) factors that play important roles in cap-independent translation. Here we have investigated the IRNA-binding proteins that might be involved in cap-independent translation in the yeast *Saccharomyces cerevisiae*. We have identified Zuotin, a DnaJ chaperone protein similar to mammalian HSP-40 chaperone, which interacts strongly with IRNA. Using *ZUO1*-deleted *S. cerevisiae*, we demonstrate a preferential requirement of Zuo1p for cap-independent translation mediated by the 5' untranslated region of the yeast TFIID mRNA. Further studies using *zuo1Δ S. cerevisiae* complemented with various Zuo1p mutants indicate that the DnaJ domain of Zuo1p, known to influence its interaction with HSP-70, significantly affects cap-independent translation. These results demonstrate for the first time a role for an established chaperone protein in cap-independent translation of a cellular mRNA.

Keywords

Cap-independent translation; IRES-mediated translation; yeast; TFIID 5'UTR; Zuotin; DnaJ molecular chaperone; yeast homolog of mammalian HSP-40

Introduction

Although it has been known for a while that cell-free extracts from yeast are capable of translating IRES elements (1,2), recent *in vivo* studies have provided more convincing evidence of cap-independent translation in yeast. Zhou *et al.* have shown that the leader regions of yeast *YAP1* and *TIF4631* can support IRES-mediated translation in logarithmically growing yeast (3,4). Paz *et al.* have reported a 140 nt long RNA from the *lacI* gene of *E. coli* which could act as an IRES element in yeast cells (5). The IRES element of cricket paralysis virus functions in yeast by a mechanism that is independent of initiator tRNA/eIF-2 (6). In addition, the Hap4 protein and a truncated form of the Ure2 protein are synthesized by internal initiation of translation in yeast (7,8).

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We previously reported a small RNA (called IRNA) from the ABYS1 strain of *S. cerevisiae*, which inhibited poliovirus and HCV IRES-mediated translation both *in vivo* and *in vitro* without interfering with cap-dependent translation of cellular mRNA (9–11). Subsequent studies revealed that IRNA acted by sequestering a number of cellular RNA binding proteins including the La protein (Lupus autoantigen), which appears to be important for viral IRES-mediated translation (10–13). Because IRNA interacts with transacting mammalian proteins involved in cap-independent translation, we reasoned that one or more yeast proteins capable of interacting with IRNA might be involved in cap-independent translation in yeast. We report here identification of Zuotin (Zuo1p), a ribosome-associated RNA binding protein from yeast, which strongly interacts with IRNA. Zuo1p has been classified as the yeast homologue of DnaJ chaperone protein similar to mammalian HSP-40 chaperone (14,15). The HSP-40 (Dna J) in association with HSP-70 (DnaK) and Ssz1p, mediate translocation of newly made proteins to various cellular compartments (16). Using *ZUO1*-deleted yeast, we demonstrate that wt Zuo1p significantly stimulates TFIID 5'UTR-mediated translation from a bicistronic RNA *in vivo*. We also demonstrate that the highly conserved DnaJ domain of Zuo1p plays an important role in cap-independent translation. Additionally, although the yeast TFIID 5' UTR was previously shown to act as a functional IRES element *in vitro* in yeast translation extract (1), we demonstrate here that it can also mediate cap-independent translation in living yeast cells.

Materials and methods

Yeast strains

The *Saccharomyces cerevisiae* strain ABYS1 (*MATa pra-1 prb-1 prc-1 cps1-3 ade1*) used for Zuo1p purification was kindly provided by Dr. David Meyer (University of California, Los Angeles). The wt strain BY4729 (*his3 trp1 ura3*) was used to generate BY4729 *zuo1Δ*: TRP1 yeast.

Plasmid construction

The GST-Zuo1 recombinant plasmid was constructed by amplification of *ZUO1* from the ABYS1 genomic DNA using the forward primer, CCGCCCCGCATATGTTTTCTTTACCTACCCTAACCTCAGACATC, and reverse primer GCCATGGGATCCCACGAAGTAGAACAACAACAAGCTGGATGGTAG with *NdeI* and *BamHI* sites and cloned into the corresponding sites of the pESP3 expression vector (Stratagene). The wild type and deleted fragments of *ZUO1* were made by PCR using appropriate primers and cloned into phyb/Zeo vector, which was derived from phyb-Lex/Zeo (Invitrogen). A 790bp fragment of *TRP1* gene containing *StuI* and *KpnI* sites was inserted between nt 431 and 539 of the coding region of *ZUO1*. The *BamHI* and *EcoRI* digested DNA was used to transform diploid *S. cerevisiae* BY4729. Standard techniques were used for yeast transformation, sporulation and tetrad dissection (17). The disruption of *ZUO1* was checked by digestion of DNA with *EcoRI/XhoI*, which generates a 2.0kb fragment containing the *TRP1* gene instead of a 1.3kb fragment containing the intact *ZUO1* gene. The BY4729 *zuo1Δ*: TRP1 cells were transformed with wt or mutant *ZUO1* plasmids and the positive transformants were isolated by selection with zeocin.

The bicistronic construct containing the coding sequences of CAT and luciferase flanked by the TFIID 5'UTR was constructed using the pYES2 vector under *GAL1* promoter (Invitrogen). A stable stem-loop (SL) structure (18) was inserted between the stop codon of CAT and the TFIID 5'UTR. BY4729 strain of yeast was transformed with pYES2-CAT-(SL)-TFIID 5'UTR-Luc and transformants were obtained by *URA3* selection.

Deletion of GAL1 promoter from bicistronic plasmid

The *Age I* and *Hind III* flanking DNA sequence (431 bp) containing both the *GAL1* and T7 promoters was deleted by digestion with restriction enzymes *Hind III* and *Age I* followed by end-filling by Klenow polymerase. The blunt-ended product was ligated with the *Hind III* and *Age I* digested plasmid using DNA ligase. The sequence of the *GAL1* promoter-deleted plasmid was verified by DNA sequencing.

Site-directed mutagenesis

The TFIID 5'-UTR (19) and the DnaJ domain of *ZUO1* was mutated by 'QuikChange Site-Directed Mutagenesis Kit (Stratagene) using appropriate primers (table 1, supplementary material).

Plasmid transformation in Yeast

The pYES2 based bicistronic plasmids containing wild-type and various mutated TFIID 5'UTR were transformed chemically using *S. cerevisiae* EasyComp Transformation Kit (Invitrogen). The transformed colonies were selected auxotrophically in the minimal YC Ura⁻ plate. The yeast cells were single-colony purified from the selection plate.

Luciferase and CAT activity measurements

The cell-free yeast lysates were prepared as previously described (15). The luciferase activity was determined as per manufacturer's instructions (Promega). The CAT gene expression was monitored by either direct activity assay using a Liquid Scintillation Counting assay (Promega) or by densitometric scanning of the CAT protein following SDS-PAGE.

Preparation and fractionation of yeast ribosomal salt wash and protein purification

Ribosomal salt wash (RSW) was prepared from the ABYS1 strain grown in YPD to an OD₆₀₀ of 0.7–0.9 at 30° C as previously described (15). The 0.5 M KCl RSW was dialyzed overnight against dialysis buffer (20 mM Tris pH 7.5, 100 mM KCl, 1mM EDTA, 1mM DTT and 5% glycerol). The RSW was applied to a Q-Sepharose column previously equilibrated with the dialysis buffer and proteins eluted stepwise using various concentrations of KCl in dialysis buffer. The 0.3M fraction from the Q-Sepharose column was loaded onto a Poly(U) Sepharose and bound proteins were eluted stepwise with the same buffer containing 0.2, 0.3, and 0.6 M KCl. The GST-Zuo1 recombinant protein was purified by glutathione-Agarose affinity chromatography.

Northwestern, UV-crosslink and Immunoblot analyses

One hundred to 200µg of RSW or 2–10µg of partially purified proteins were separated on a 12% SDS-PAGE, transferred to nitrocellulose and allowed to renature in renaturation buffer (10mM Tris, pH 7.5, 50 mM NaCl, 1mM MgCl₂) at 4°C. The nitrocellulose filter was incubated with 200,000 cpm of 32P labeled IRNA or TFIID 5' UTR probe in renaturation buffer for 60 min at room temperature. After washing three times in 10 mM Tris, pH 7.5, 50 mM NaCl, membrane was dried and exposed to X-ray film. For Western analysis, following blocking for 1 hr with 1% blocking reagent, the blot was probed with a polyclonal antibody to Zuo1p for 1 hr. The secondary antibody was conjugated with Dig-label and the immunoblot was developed using the chemiluminescence detection system (Pierce). Northern blot and UV-crosslink analyses were performed as previously described (20).

Results

TFIID 5' UTR can support cap-independent translation in living yeast cells

To examine cap-independent translation from the TFIID 5'UTR, wt BY4729 cells were transformed with a bicistronic reporter, pYES2-CAT-(SL)-TFIID 5'UTR-Luc under the *GALI* promoter (Fig. 1A). Upon induction by galactose, the *GALI* promoter is expected to drive expression of a single mRNA encompassing both cistrons. The first cistron, CAT, is translated via cap-dependent translation, while the second cistron, Luc, should be translated in a cap-independent manner due to the presence of the TFIID 5' UTR. A highly stable stem-loop (SL) structure was placed just upstream of the 5'UTR to discourage reinitiation following translation from the first cistron (18). After induction with galactose, luciferase and CAT activities were measured in cell-free extracts. The presence of the TFIID 5'UTR was found to be almost absolutely essential for synthesis of luciferase; substitution of the TFIID 5' UTR with the multiple cloning site or an unrelated sequence of similar length resulted in a 40 fold reduction of luciferase activity in wt cells (Fig. 1B). Both constructs, however, directed synthesis of almost equal amounts of the CAT protein (Fig. 1B, inset). The synthesis of both luciferase and CAT was dependent on induction by galactose (Fig. 1B). These results suggest that the TFIID 5'UTR is able to confer cap-independent synthesis of luciferase from the second cistron in living yeast cells.

As a first step to characterize the TFIID 5'UTR, 6nt stretches of the wt 5'UTR sequence was substituted with the *AgeI* restriction site (accggt) (Fig. 1A). All 5'UTR mutants were found to be defective in cap-independent translation to varying degrees compared to the wt 5'UTR (Fig. 1C). Mutant M1 displayed the lowest cap-independent translation activity, which was approximately 1–2% of the wt control. Mutants M2 and M5 were significantly defective in TFIID 5'UTR-mediated translation (4–5 fold), while translation from mutants M3, M4, and M6 was reduced by approximately 47, 40, and 30%, respectively compared to the wt 5' UTR. A single species of RNA having an appropriate size of the bicistronic RNA was found in both wt and mutant transformed cells induced by galactose, but not in the uninduced control (Fig. 1C, inset). These results suggest that almost the entire 5' UTR is required for efficient synthesis of luciferase from the TFIID 5'UTR and the 6 nucleotide sequence at position 11–16 of the TFIID 5'UTR is highly critical for cap-independent translation.

TFIID 5'UTR does not contain a cryptic promoter

To determine whether the TFIID 5'UTR contains a cryptic promoter, which could contribute to synthesis of luciferase, the upstream *GALI* promoter was deleted from the bicistronic plasmid. Yeast was transformed with the wt or *GALIpΔ* constructs, and the levels of luciferase and CAT were determined following induction with galactose. While both luciferase and CAT proteins were synthesized in yeast transformed with the wt construct in all four experiments, both the CAT protein and luciferase activity were completely absent in yeast transformed with the *GALIp*-deleted plasmid (Fig. 2A and B). Northern analysis showed no detectable synthesis of bicistronic RNA in *GALIpΔ* plasmid transformed cells compared to those transformed with the wt construct (Fig. 1C). These results suggest that the TFIID 5'UTR-mediated translation does not result from a functional cryptic promoter within the TFIID 5'UTR under our assay conditions.

Identification of yeast proteins that interact with IRNA

To identify IRNA binding proteins in yeast that might be involved in cap-independent translation, ribosomal salt wash (RSW) was purified through a Q Sepharose column. Bound proteins were eluted stepwise with buffer containing 0.3, 0.6, 0.75 and 1.0 M KCl and analyzed by Northwestern blotting using ³²P labeled IRNA as the probe. A polypeptide with an apparent molecular weight of 60kDa was found in the 0.3M fraction that bound labeled IRNA relatively

strongly (Fig. 3B, lane 4). This fraction was further purified by Poly(U)- Sepharose chromatography. The majority of the 60kDa protein eluted in the 0.3M KCl fraction from the Poly (U) Sepharose column co-migrated with the labeled 60 kDa polypeptide in the Northwestern blot (lane 8, Figs. 3A and B). The majority of the low molecular weight IRNA-binding proteins was not retained by Q-Sepharose and found in the flow-through fraction (Fig. 3B, lane 3). The 60 kDa IRNA-binding protein was excised from the gel (Fig. 3C), and the amino acid sequence of an N-terminal 19 amino acid peptide of the 60 kDa protein was determined (Fig. 3E).

Comparison of this sequence with that of the known proteins from the yeast data base revealed a striking homology with an yeast protein called Zuotin, except for the three serine residues whose identity could not be confirmed during the first sequencing run (Figs. 3D and E). A second sequencing analysis confirmed the presence of three missing serine moieties at positions 3, 9 and 17 in the N-terminal peptide. Also, a polyclonal antibody to Zuo1p specifically reacted with the 60 kDa, IRNA-binding polypeptide (data not shown). These results strongly suggested that the 60 kDa IRNA-binding protein was Zuotin.

Expression, purification and RNA binding activity of Zuotin

We expressed and purified GST-Zuo1p and examined its RNA binding ability. The purified GST-Zuo1p showed a major band at 87 KDa as expected (Fig. 3F, lane 2). GST-Zuo1p, but not GST, bound both radiolabeled IRNA (lanes 1 and 2, Fig. 3G) and TFIID 5'UTR (Fig. 3G, lanes 3 and 4) in a UV cross-link assay. The binding of TFIID 5'UTR to GST-Zuo1p was more efficient (~4 fold) compared to IRNA (compare lane 4 with lane 2, Fig. 3G). UV-crosslink analysis using labeled TFIID 5' UTR and various concentrations of unlabeled competitor RNA showed that the unlabeled homologous RNA was a better competitor than a heterologous RNA (Fig. 3H). Thus, Zuo1p appears to interact specifically with the TFIID 5' UTR.

Zuotin stimulates IRES-mediated translation in living yeast cells

To examine the role of Zuo1p in cap-independent translation, the *ZUO1* gene was deleted from BY4729 yeast. The *ZUO1*-deleted (*zuo1Δ*) yeast displayed slow growth phenotype as reported previously (14, 15, and data not shown). *zuo1Δ* cells were stably transformed with two plasmids: the bicistronic reporter pYES2-CAT-(SL)-TFIID 5'UTR-Luc and the wt or various *ZUO1* mutants (Fig. 4A). These cell lines constitutively express wt or various Zuo1p mutants, and synthesize the reporter mRNA when induced by galactose. We arbitrarily assigned a value of 1 to both CAT and Luc synthesized in *zuo1Δ* cells transformed with the bicistronic reporter construct, and the ratio of Luc to CAT was plotted as an indicator of TFIID 5'UTR-mediated translation. There was significant stimulation of TFIID 5'UTR-mediated translation (~3.5 fold) in *zuo1Δ* cells expressing wt Zuo1p compared to the control *zuo1Δ* cells (Fig. 4B). Deletion of neither the N-terminal 101 amino acids (mutant 102-433) nor the C-terminal 149 amino acids (mutant 1-284), which includes the charged region responsible for interaction with RNA, did not affect the Luc to CAT ratio significantly compared to the wt protein (Fig. 4B). These results suggested that Zuo1p terminal sequences were not absolutely necessary for stimulation of TFIID 5'UTR-mediated translation. In contrast, deletion of the N-terminal 165 amino acids (mutant 166-433), which removes the DnaJ domain required for protein-protein interaction, almost totally abrogated TFIID 5' UTR-mediated translation bringing back the Luc to CAT ratio close to 1. Deletion of the DnaJ domain (Δ 111-165) also consistently reduced Luc to CAT ratio to 1. These results suggest that the DnaJ domain of Zuo1p plays an important role in cap-independent translation from the TFIID 5'UTR. Finally, deletion of the charged region (Δ 285-364) did effect cap-independent translation modestly compared to the wt Zuo1p control (Figs. 4A and B). This reduction could be due to a structural change in the protein since the 1-284 mutant, which lacks the entire charged region (285-364) was as active as the wt protein in stimulating TFIID 5'UTR-mediated translation. Northern analyses of total RNA isolated

from these cells showed that the majority of the RNA migrated with an expected size of the bicistronic reporter RNA in *zuo1Δ* cells expressing wt Zuo1p or various Zuo1p mutants (Fig. 4C). Western analyses showed that the wt and various Zuo1p mutants were expressed and stable under the experimental conditions (Fig. 4B, inset). There was no significant difference in the rate of degradation of luciferase and CAT up to 4 h after treatment with cycloheximide in *zuo1Δ* BY4729 cells (data not shown). These results suggest that Zuo1p preferentially stimulates TFIID 5' UTR-mediated translation *in vivo* and the DnaJ domain of Zuo1p plays an important role in TFIID IRES-mediated translation.

Interaction of the DnaJ domain of Zuo1 with HSP-70 (DnaK) is important for cap-independent translation from the TFIID 5'UTR

Previous studies (21–24) have identified two highly conserved regions within the J domain in the *E. coli* DnaJ protein, which appear to be important for its interaction with the cognate DnaK (HSP-70): one localized to helix II (amino acids residues 18–32, Fig 5A) and the adjacent loops, including the conserved HPD tripeptide (amino acid residues 33–35, Fig. 5A), and another near the distal end of the J domain that includes the QKRAA motif (Fig. 5A). We introduced mutations in these regions to examine the role of the J domain of Zuo1p in cap-independent translation from the TFIID 5'UTR. Cap-independent translation was reduced by ~5 fold in *ZUO1*-deleted yeast transformed with ΔJ deletion mutant compared to the wt *ZUO1 DNA* (Fig. 5B). The Zuo1p having triple alanine substitution of the first three amino acids (KYH, residues 31–33) that includes the His33 of the HPD tripeptide (mutant J1, Fig. 5B) was highly defective in promoting cap-independent translation, although cap-dependent translation was almost unaffected (Fig. 5B). Both cap-independent and cap-dependent translation was reduced significantly in the J2 mutant where the PDK residues (amino acids 34–36) were substituted by 3 alanines. The Luc to CAT ratio, however, was reduced by 40% compared to the wt control. The point mutant J3 (D35N) was significantly defective in cap-independent translation compared to Zuo1p with wt J domain. The two point mutants, J5 (K62E) and J4 (R63S) were also significantly defective with 77 and 57% reduction in cap-independent translation, respectively compared to the wt J domain. Western blot analysis showed comparable levels of wt and various mutant proteins in the transformed yeast (data not shown). Identical point mutations in the J domain (J5 and J4) have been previously shown to affect DnaJ-DnaK interaction significantly (22,23). These results suggest that protein-protein interaction involving the Zuo1p DnaJ domain and HSP-70 plays an important role in cap-independent translation mediated by the TFIID 5'UTR in yeast.

Discussion

We present genetic evidence, which suggests a role of Zuo1p, a DnaJ molecular chaperone, in TFIID 5'UTR-mediated, cap-independent translation in the yeast *Saccharomyces cerevisiae*. Zuo1p was identified by its ability to interact with a small yeast RNA (IRNA), which was shown previously to block polio and hepatitis C virus IRES-mediated translation. A similar approach had previously identified two mammalian proteins, La and nucleolin, both of which stimulate viral IRES-mediated translation (10,25).

How does Zuo1p preferentially stimulate cap-independent translation? Zuo1p could directly effect TFIID 5'UTR-mediated translation by binding to TFIID mRNA and promoting protein-protein interaction leading to ribosome recruitment. Alternatively, it could act indirectly by affecting another protein, which may be required for cap-independent translation. For example, a significant drop in the effective concentration of a limiting factor required specifically for cap-independent translation due to lack of proper protein folding or translocation in *Zuo1Δ* cells could lead to selective decrease in cap-independent translation. Further genetic and

biochemical studies should facilitate the elucidation of the precise role of Zuo1p in cap-independent translation.

The relative stimulation of cap-independent over cap-dependent translation in *zuo1Δ* cells is almost absolutely dependent on the DnaJ domain (Figs. 4 and 5) suggesting that Zuo1 protein-protein interaction play an important role in cap-independent translation. Moreover, single amino acid changes within two regions of the J domain, known to interfere with its interaction with HSP-70 (22,23), significantly affect cap-independent translation (Fig. 5). Thus, the interaction between Zuo1p (DnaJ) and HSP-70 (DnaK) appears to be important for cap-independent translation from the TFIID 5' UTR *in vivo*.

Although the 1-284 and 102-433 mutants were able to complement translation in *zuo1Δ* cells, none of the Zuo1p mutants could complement the growth defect in these cells (data not shown). This suggests that translation is not the only activity that is compromised by *ZUO1* deletion and domains other than DnaJ and RNA binding are likely involved in other important functions of this protein. These results are consistent with previous data, which demonstrated that deletion of the N-terminus up to the J domain (166-433) resulted in a stable but non-functional protein, which was able to bind RNA and associate with ribosome, but failed to complement the growth defect in *zuo1Δ* cells (15).

The TFIID 5'UTR was previously shown to mediate cap-independent translation in yeast cell-free extracts (1). The results presented here demonstrate that the TFIID 5'UTR can also promote cap-independent translation in living yeast cells, and most probably acts as an IRES element. We cannot completely rule out the possibility that translation *in vivo* from the second cistron (Luc) of the reporter RNA could occur by other modes of translation initiation such as reinitiation or ribosome shunting. However, the fact that a number of substitution mutations in the TFIID 5'UTR significantly affects synthesis of luciferase is consistent with internal initiation of translation (Fig. 1). Future studies involving point mutations within the TFIID 5' UTR should help understand the role of the TFIID 5'UTR in cap-independent translation.

A previous report by Hecht *et al.* (26) has suggested the presence of a cryptic promoter situated between -188 to -103 (spanning mutants M4 and M5, Fig. 1) in the TFIID 5'UTR that could explain increased production of luciferase from a monocistronic transcript synthesized from the cryptic promoter. Alternatively, the bicistronic reporter DNA may have been integrated downstream of an unrelated promoter in stably transformed cells, and transcription from this promoter could possibly generate an mRNA encoding luciferase. Both scenarios, however, seem highly unlikely for the following reasons: first, the majority of RNA synthesized after galactose induction migrates with a size consistent with the bicistronic RNA (Figs. 1C and 4C). Moreover, no RNA that hybridizes to the labeled luciferase probe is detected in uninduced cells (Fig. 4C), and similarly only background luciferase activity is detected in uninduced cells ((Fig. 1). The synthesis of luciferase encoding transcript(s) by either mechanism should not depend on induction by galactose. Secondly, the presence of a cryptic promoter at -188 to -103 could not explain why mutant M1 had lost almost all IRES activity. Finally, we were unable to detect any significant luciferase activity in yeast transformed with a bicistronic construct lacking the *GALI* promoter (Fig. 2). Thus, it seems highly unlikely that transcription from either a cryptic promoter within the TFIID 5' UTR or an unknown yeast promoter is contributing to luciferase synthesis.

How does IRNA regulate cap-independent translation in yeast? Previous studies in mammalian cells that constitutively express IRNA have shown that it could possibly regulate viral IRES-mediated translation by sequestering transacting factors (such as La) that stimulate viral 5' UTR-mediated translation (10,11). Although the human La protein does not stimulate HCV IRES-mediated translation in yeast (27), IRNA may regulate cap-independent translation in

yeast by sequestering Zuotin. It is worth mentioning in this context that we have detected other IRNA-binding proteins than Zuotin in the BY4729 strain of yeast (Fig. 4B, lane 3). It remains to be seen whether these proteins play any role in cap-independent translation in yeast.

Zuotin, a cytosolic J type chaperone, is almost quantitatively associated with ribosome. Another ribosome-associated yeast DnaJ chaperone, Sis1p, has been implicated in translation initiation (28). However, there is no direct evidence suggesting a functional role of Sis1p in translation. To our knowledge this is the first report, which demonstrates a role of Zuo1p in cap-independent translation from a cellular mRNA in yeast. Further genetic and biochemical studies should lead to a better understanding of the role of Zuo1p in cap-independent translation in yeast.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

TFIID 5' UTR mediates cap-independent translation in wt BY4729 cells.

(A) Schematic representation of the pYES2-CAT-(SL)-TFIID 5'UTR-Luc bicistronic construct and the TFIID 5' UTR (nt 1-272) mutagenesis strategy are shown. The sequences in capital letters represent the wild type, and those with small letters indicate the 5'UTR mutants. The name of each 5'UTR mutant is indicated below the substitution sequence, accggt. (B) Luciferase and CAT activities were measured after 4h induction with galactose from uninduced (U) and galactose-induced (I) wt BY4729 cells transformed with pYES2 CAT (SL) TFIID 5' UTR Luc (bars 1 and 2) and pYES2 CAT (SL) Luc (bar 3). One hundred percent Luc activity is 347,532 light units. The inset shows the SDS-PAGE analysis of CAT polypeptide from the

same experiment. (C) Effect of TFIID 5'UTR substitution mutagenesis on cap-independent translation. Luciferase and CAT activities were measured from 40 μ g of extract from BY4729 cells stably transformed with the wild type (WT) or various 5' UTR mutants (M1-M6). TFIID 5'UTR-mediated translation, as measured by Luc to CAT ratio was determined from 2 separate transformations in duplicates with very similar results. The inset in figure 1C shows Northern analysis of the bicistronic RNA using a luciferase probe for the wild type uninduced (U) and induced (WT and mutants M1-M6) BY4729 cells from the same experiment as in figure 1C. Ribosomal RNA from the same ethidium bromide stained gel is shown as loading control.

Fig. 2.

Evidence that a cryptic promoter is not responsible for TFIID 5'UTR IRES activity. (A and B) Yeast cells were transformed with either the Wt or *GAL1* promoter deletion (*GAL1pΔ*) constructs. Luc and CAT were measured from four (1–4) separate experiments. (C) Northern blot of the bicistronic RNA and actin RNA (internal control) from one experiment is shown.

Fig. 3.

Purification of IRNA-binding proteins from ABYS1 yeast. Various fractions (2–4 µg) as indicated (lanes 2–9) were either silver stained (A), or probed with ^{32}P -labeled IRNA in a Northwestern blot (B). The Molecular weight markers and RSW were analyzed in lanes 1 and 2, respectively. The IRNA-binding polypeptide indicated by the asterisk in Fig. 3A (lane 8) was excised and eluted from 15 identical lanes, pooled and concentrated, and a 5 µl aliquot analyzed by SDS-PAGE (C). The amino acid sequence of N-terminal 19 amino acid long peptide of Zuo1p (E) is compared with the Zuo1 N-terminal sequence from the yeast database (D). (F–H) Silver stain of 1 µg affinity purified GST-Zuo1 fusion protein from uninduced (lane 1) and induced (lane 2) cells (F). UV cross-link analysis of 200 ng GST-Zuo1 (lanes 2 and 4)

or GST (lanes 1 and 3) with 200,000 cpm each of ^{32}P -labeled IRNA (lanes 1, 2) and TFIID 5' UTR (lanes 3, 4) (G). Molecular weight markers are shown in lane M. UV cross-link analysis of GST-Zuo1- ^{32}P TFIID 5' UTR complex formed in the absence (lane 2) or presence of 100, 200 and 300 fold molar excess of cold TFIID 5'UTR (lanes 3, 4 and 5) or an unrelated RNA of similar length (lanes 6, 7 and 8) (H). Lane 1 is same as lane 2 except GST was used instead of GST-Zuo1 (H).

Fig. 4.

Dependence of TFIID 5'UTR-mediated cap-independent translation on Zuo1p DnaJ domain (A) Schematic representation of the domain structure of wt and various Zuo1p mutants. (B) TFIID IRES-mediated translation was determined from 3 separate transformations. The Luc to CAT ratio from *Zuo1Δ* cells was arbitrarily given a value of 1. The inset shows Zuo1p Immunoblot analysis from *Zuo1Δ* cells (lane 1)-, and those transformed with wt (lane 2)- or various *ZUO1* mutant (lanes 3–7) plasmids. The estimated molecular weights of wt Zuo1p and various mutants are as follows: wt (60kDa), 102-433 (45 kDa), 166-433 (37 kDa), Δ111-165 (52 kDa), Δ285-364 (49 kDa), and 1-284 (39 kDa). (C) Northern analysis of total RNA from *Zuo1Δ* cells transformed with the bicistronic construct pYES2 CAT (SL) TFIID5'UTR Luc (lane 2) or transformed with both the bicistronic plasmid and the wt or various Zuo1p mutants as indicated (lanes 3–8). RNA recovered from galactose induced (lanes 2–8) and uninduced (lane 9) *Zuo1Δ* cells and *in vitro* transcribed bicitronic RNA (lane 1) were analyzed. In the lower panel Yeast *cdc19* RNA was analyzed from total RNA isolated from transformed cells as loading control.

each bar shows the actual numbers of cap-independent (numerator) versus cap-dependent (denominator) translation for wt and various J mutants from one representative experiment.