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A *Saccharomyces cerevisiae* homologue of mammalian translation initiation factor 4B contributes to RNA helicase activity

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The *TIF3* gene of *Saccharomyces cerevisiae* was cloned and sequenced. The deduced amino acid sequence shows 26% identity with the sequence of mammalian translation initiation factor eIF-4B. The *TIF3* gene is not essential for growth; however, its disruption results in a slow growth and cold-sensitive phenotype. *In vitro* translation of total yeast RNA in an extract from a *TIF3* gene-disrupted strain is reduced compared with a wild-type extract. The translational defect is more pronounced at lower temperatures and can be corrected by the addition of wild-type extract or mammalian eIF-4B, but not by addition of mutant extract. *In vivo* translation of β-galactosidase reporter mRNA with varying degree of RNA secondary structure in the 5′ leader region in a *TIF3* gene-disrupted strain shows preferential inhibition of translation of mRNA with more stable secondary structure. This indicates that Tif3 protein is an RNA helicase or contributes to RNA helicase activity *in vivo*.
Key words: RNA binding domain/RNA helicase/RNA secondary structure/translation initiation factor/*Saccharomyces cerevisiae*

Introduction

Initiation of translation consists of a number of consecutive biochemical reactions in the pathway of protein synthesis and is an important site for regulation of gene expression (for reviews see Moldave, 1985; Pain, 1986; Müller and Trachsel, 1990; Hershey, 1991). The aim of these reactions is to position the ribosome at an initiator AUG on the mRNA so that polypeptide elongation can begin. In eukaryotes, the predominant way by which ribosomes find the initiator AUG is movement from the 5′ m^7GpppN cap structure in the 5′ to 3′ direction along the mRNA. This process was termed scanning (Kozak and Shatkin, 1978; for a review see Kozak, 1989). Ribosomal subunits recognize proteins associated with the cap structure of the mRNA (for reviews see Rhoads, 1988; Sonenberg, 1988) and bind on to or near the cap structure (Kozak, 1980). From there they move along the mRNA in an ATP hydrolysis-requiring process (Kozak, 1980) and usually recognize the first AUG codon as the initiator codon (Kozak, 1989). It is believed that scanning is performed by 40S ribosomal subunits carrying initiator methionyl-tRNA (Met-tRNAi), since Met-tRNAi is found associated with 40S ribosomal subunits not yet bound to mRNA (Darnbrough *et al.*, 1973; Levin *et al.*, 1973) and mRNA binding to ribosomes requires Met-tRNAi (Trachsel *et al.*, 1977; Benne and Hershey, 1978). Met-tRNAi (Cigan *et al.*, 1988) and the sequences flanking the AUG codon (Kozak, 1989) are involved in initiation codon recognition. Local RNA secondary structure is melted during scanning or, if it cannot be melted, impedes the movement of the ribosome on the mRNA (for a review see Hinnebusch and Liebman, 1991).

This complex sequence of reactions is catalysed by eukaryotic translation initiation factors (eIFs, for reviews see Moldave, 1985; Pain, 1986; Rhoads, 1991). A cap-binding protein complex, eIF-4F, recognizes the cap structure through the subunit eIF-4E (Moldave, 1985; Pain, 1986; Rhoads, 1988, 1991; Sonenberg, 1988) and together with the factors eIF-4A and eIF-4B promotes local RNA unwinding to allow the binding of the 40S−Met-tRNAi complex. Unwinding of RNA secondary structure downstream of the ribosome binding site is then catalysed either by ribosome-associated proteins or by an RNA helicase that acts independently of the ribosome. *In vitro* RNA helicase assays indicate that this helicase contains at least two factors: eIF-4A and eIF-4B (Rozen *et al.*, 1990). However, the additional participation in RNA secondary structure melting during scanning of eIF-4F and the recently described factors Ssl1 and Ssl2 (Gulyas and Donahue, 1992; Yoon *et al.*, 1992) is possible.

The process of translation initiation appears to be very conserved between eukaryotes as far apart as mammals and the yeast *Saccharomyces cerevisiae*. This conclusion is based among others on comparisons of mRNA structure, mode of translation initiation and initiation factor structure and function (for reviews see Müller and Trachsel, 1990 and references therein; Linder, 1992). Specifically, *S.cerevisiae* ribosomes, like their mammalian counterparts, initiate translation at the first AUG downstream of the cap structure of mRNA (Sherman and Stewart, 1975) and translation is influenced by RNA secondary structure in the 5′ untranslated region of mRNA (Baim *et al.*, 1985; Baim and Sherman, 1988). For these reasons and because *S.cerevisiae* offers the possibility to combine biochemical studies with powerful genetic approaches, we and others have begun to use this organism to study the mechanism and regulation of mRNA binding to ribosomes.

The genes encoding translation initiation factors involved in mRNA binding to the ribosome, such as eIF-4A (Blum *et al.*, 1989; Linder and Slonimski, 1988, 1989), eIF-4E (Altmann *et al.*, 1985, 1987), eIF-4F (Goyer *et al.*, 1989, submitted) and Ssl1 and Ssl2 (Gulyas and Donahue, 1992; Yoon *et al.*, 1992) were cloned from *S.cerevisiae*. The gene encoding eIF-4B, however, an initiation factor that supposedly plays a central role in mRNA binding to

ribosomes and RNA secondary structure unwinding, has not yet been identified in *S.cerevisiae*.

Here, we report the cloning and initial characterization of the yeast *S.cerevisiae* gene *TIF3* encoding a translation initiation factor with sequence homology to mammalian eIF-4B.

Results

Cloning of the TIF3 gene

In the process of screening *S.cerevisiae* genomic and cDNA libraries with a polyclonal antibody directed against yeast eIF-4E, we isolated eIF-4E cDNA clones (Altmann *et al.*, 1987) and additional DNA sequences (Altmann *et al.*, 1989; Goyer *et al.*, 1993). Among them was a 123 bp genomic DNA fragment (sequence between the arrows in Figure 1), which was confirmed later to be part of the *TIF3* gene of *S.cerevisiae*. This genomic DNA fragment was inserted into the plasmid Yip5. The plasmid was linearized at the *Mlu*I restriction site in the DNA insert and transformed into a diploid *S.cerevisiae* strain to allow insertion by homologous recombination (for details see Materials and methods). After sporulation of the diploid strain and tetrad dissection, haploid cells carrying the inserted plasmid were selected. They showed a slow growth and cold-sensitive phenotype (see below). Southern blot analysis confirmed the integration of the plasmid into the *TIF3* gene (results not shown). Genomic DNA from one of these haploid strains (MT200D) was isolated, the two parts of the interrupted gene with flanking sequences recovered and ligated to restore the intact gene (see Materials and methods). A 4.6 kb *Eco*RI DNA fragment which complemented the slow growth and cold-sensitive growth phenotype of strain MT200D was subcloned and sequenced.

Characteristics of the TIF3 gene and the gene product

The genomic *Eco*RI DNA fragment carries an open reading frame encoding a protein of 436 amino acids with a calculated molecular weight of 48.5 kDa (Figure 1). A comparison of this amino acid sequence with sequences in the SwissProt data bank revealed 50% similarity and 26% identity (115 perfect amino acid matches) with mammalian translation initiation factor 4B (eIF-4B, Milburn *et al.*, 1990). An alignment of the two sequences is shown in Figure 2. Regions of homology are found throughout the two proteins except for the 45 C-terminal amino acids of eIF-4B (total length 611 amino acids). Both proteins contain the motifs RNP2 (sequence AVINNI for yeast Tif3, sequence AFLGNL for human eIF-4B) and RNP1 (sequence KGNAFVTL for yeast Tif3, sequence KGFGYAEF for human eIF-4B) spaced by 38 amino acids in Tif3 and 33 amino acids in eIF-4B (Figure 2). These sequence motifs were shown to lie adjacent to each other on two β-strands in a $\beta\alpha\beta\beta\alpha\beta$ structural domain in several RNA-binding proteins (for a review see Kenan *et al.*, 1991) and to be directly involved in RNA recognition (Görlach *et al.*, 1992). Neither the RNP2 nor RNP1 sequences in Tif3 and eIF-4B are very similar. However, they show the typical spacing of hydrophobic amino acid residues and the conservation of certain key residues (Kenan *et al.*, 1991). Comparison of the RNP2 sequences of Tif3 and eIF-4B with RNP2 sequences from 54 different sources (Kenan *et al.*, 1991) reveals that apart from one exception only Tif3 and eIF-4B have an alanine residue at position 1. The significance of this observation is not clear at present. Secondary structure

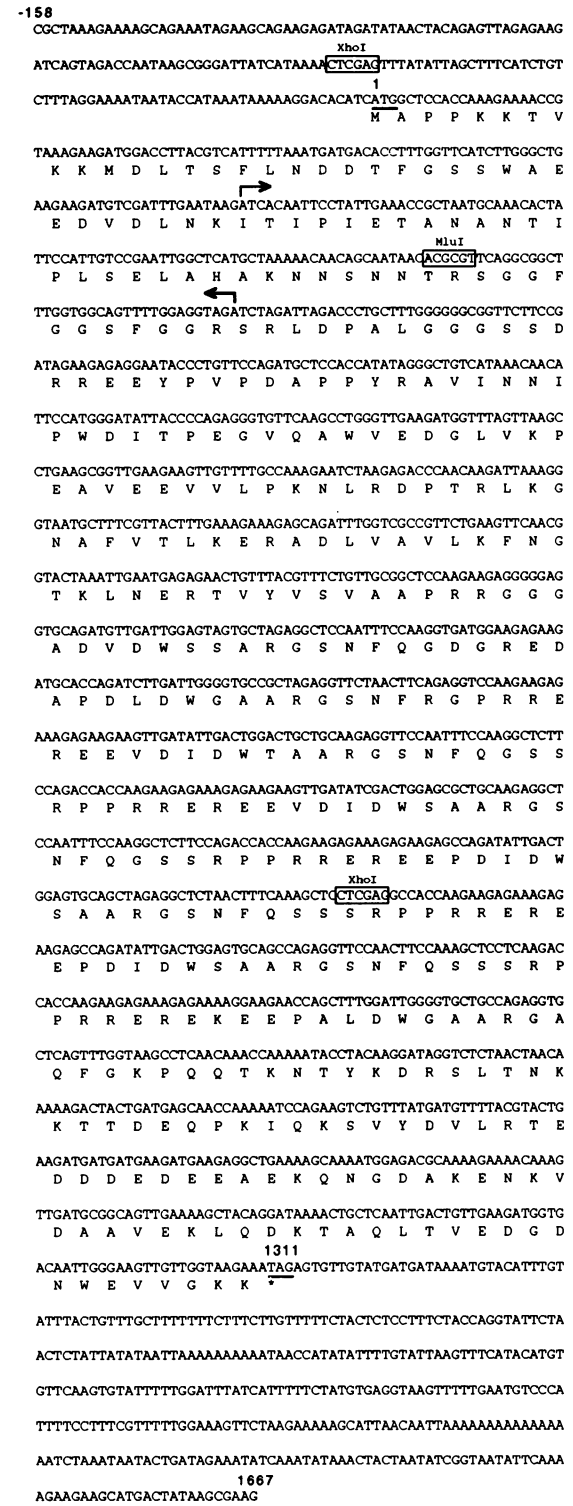

Fig. 1. DNA sequence and derived amino acid sequence of the *TIF3* gene. Part of the DNA sequence of the *Eco*RI DNA fragment encoding Tif3 is shown. Both strands were sequenced as described in Materials and methods. The translation start and stop codons are underlined; numbers correspond to the beginning of the sequence presented (pos. −158), the start codon (pos. 1), the stop codon (pos. 1311) and the end of the sequence presented (pos. 1667). Arrows indicate the boundaries of the 123 bp genomic DNA fragment originally isolated from a pEX library (see Results). Restriction enzyme cleavage sites indicated are *Mlu*I and *Xho*I.

Fig. 2. Alignment of the amino acid sequences of Tif3 and eIF-4B. The amino acid sequences of yeast Tif3 (Figure 1) and human eIF-4B (Milburn *et al.*, 1990) were aligned using the program BESTFIT from the GCG software package (Devereux *et al.*, 1984) on a VAX computer. The 45 C-terminal amino acids of eIF-4B are not shown. RNP2 and RNP1 sequence motifs are boxed and the repeated sequences in the yeast protein are marked by lines with arrows and numbered 1–7.

prediction for the amino acid sequences containing RNP2 and RNP1 in Tif3 and eIF-4B (program PEPPLOT of the GCG software package) indicates that both motifs in the two proteins are potentially part of a β-strand in a $\beta\alpha\beta\beta\alpha\beta$ domain (results not shown). In Tif3, this domain is followed by a 7-fold repeat of a 26 amino acid sequence rich in basic and acidic residues (Figure 2, arrows with repeat number). Four of the repeats (repeat 3–6) are almost identical [sequence PRREREEV(P)DIDWS(T)AARGSNFQG(S)SS-RP]. The repeats 1, 2 and 7 are incomplete. A comparison of this sequence with protein sequences in the databank revealed no similarity with other sequences. In the corresponding region, eIF-4B also displays a high content of basic and acidic residues and short repeats (sequence DRYD; Milburn *et al.*, 1990).

The copy number of the *TIF3* gene in the *S.cerevisiae* genome was determined by Southern blotting analysis. Genomic DNA was digested with restriction enzymes which do not cut (*Eco*RI, *Bam*HI, *Hin*dIII and *Cla*I) or cut once (*Mlu*I) or twice (*Xho*I) in the *TIF3* gene (Figure 1) and the resulting DNA fragments hybridized with an RNA probe derived by *in vitro* transcription from the 960 bp *Xho*I fragment carrying a large part of the *TIF3* gene (see Materials and methods). The autoradiogram reveals single DNA bands digested with enzymes which do not cut in the gene and a double band for the *Mlu*I-digested DNA (Figure 3A). DNA digested with the restriction enzyme *Xho*I is expected to contain three fragments with *TIF3* gene sequences. However, since the RNA probe is derived from the *Xho*I DNA

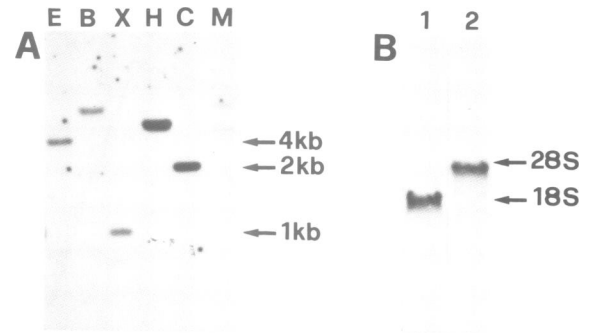

Fig. 3. Southern and Northern blot analysis of *TIF3*. (**A**) Southern blot. Genomic DNA was cut with the following restriction enzymes: E, *Eco*RI; B, *Bam*HI; X, *Xho*I; H, *Hin*dIII; C, *Cla*I; M, *Mlu*I. Note that lane M contained less DNA. (**B**) Northern blot. Lane 1, RNA from strain MT106B (wild-type); lane 2, RNA from strain MT200D (*TIF3* gene disrupted). The arrows indicate the positions on the blot of 18S and 28S ribosomal RNA. The blots were probed with a synthetic RNA corresponding to the 960 bp *Xho*I fragment of the *TIF3* gene (Figure 1; Materials and methods).

Table I. Generation time of yeast strains

Strain	Temperature (°C)	Generation time (h)
MT106B	20	4.5
	30	2.0
MT200D	20	8.5
	30	3.0

fragment, only one of the three fragments hybridizes to the probe. These data indicate that the *TIF3* gene is present in one copy per haploid genome. Northern blotting experiments show that this gene is transcribed into a 1.5 kb mRNA (Figure 3B, lane 1). Disruption of the gene by insertion of the plasmid pYip5 in strain MT200D results in the synthesis of a larger RNA (Figure 3B, lane 2).

Disruption of the *TIF3* gene by insertion of the plasmid pYip5 (strain MT200D) or by deletion of the internal 960 bp *Xho*I DNA fragment of the gene (strain RCB1) leads to a slow growth phenotype (Table I). The growth defect appears to be more pronounced at lower temperatures (cold sensitivity).

An in vitro function for Tif3 in translation

The structural similarities between Tif3 and eIF-4B prompted us to test whether the yeast protein is involved in translation. We prepared extracts from strain MT106B (wild-type) and MT200D (mutant, *TIF3* gene disrupted) and performed *in vitro* translation experiments. Extracts from both strains are active in translating total yeast mRNA (Figure 4A). At higher temperatures, the extracts behave identically and the absolute activity (^{35}S-labelled methionine incorporated per A_{260} units of extract) is very similar: mutant extract shows 75–100% of wild-type activity. Incubation of both yeast extracts at temperatures >30°C leads to progressive decline in translational activity. This is a property of yeast extracts and was discussed earlier in detail (Mandel and Trachsel, 1989). At low temperatures, however, translation in the mutant extract is more drastically reduced than in the wild-type extract. No obvious change in proteins synthesized at low temperature could be observed after fractionation of the translation products on SDS–polyacrylamide gels (results

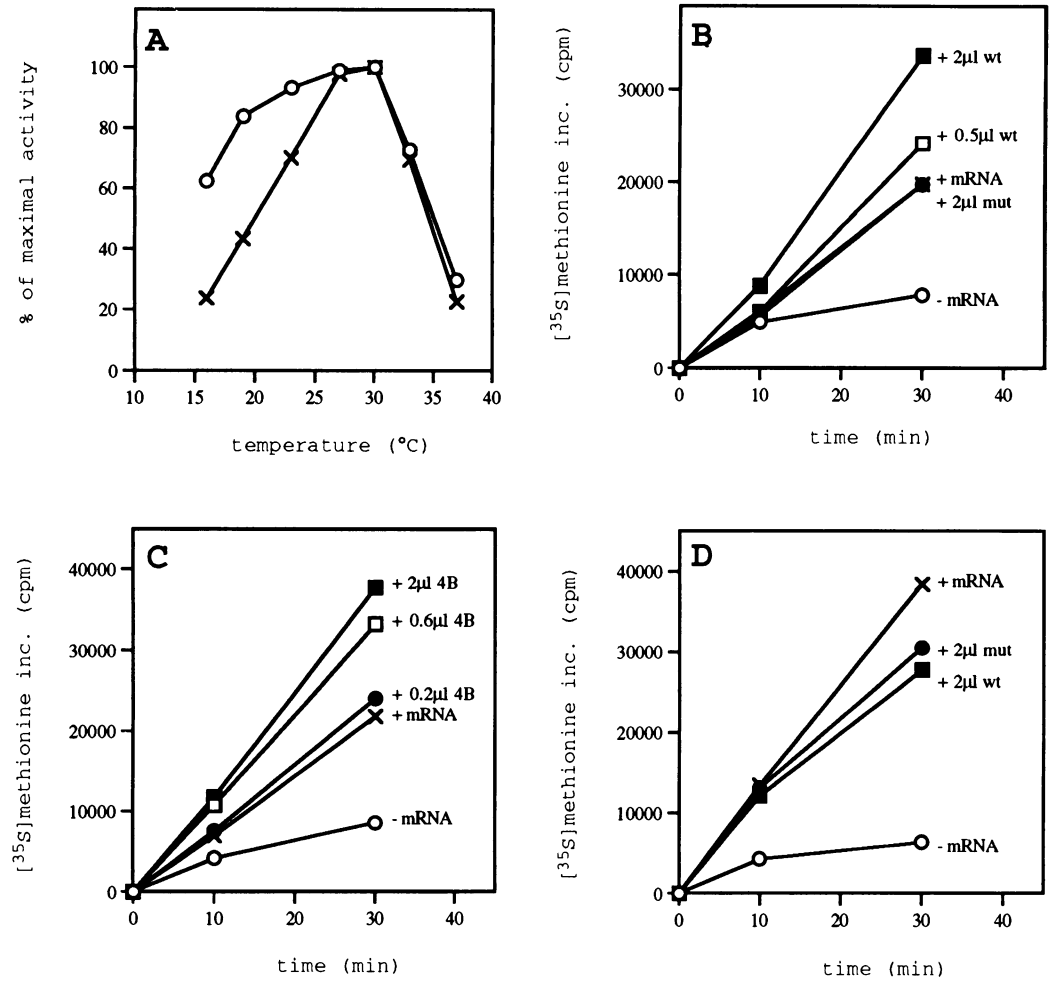

Fig. 4. *In vitro* translation of Tif3 deficient extracts. Total yeast mRNA (8 µg) was translated in 12.5 µl reaction mixtures. Aliquots of 4 µl were analysed for [^{35}S]-labelled methionine incorporation. (**A**) Incubation was for 30 min. MT106B (wild-type) extract, ×-×; MT200D (mutant) extract, ×-×. (**B**) Translation in extract MT200D (mutant). Incubation temperature was 20°C. No mRNA added, ○-○; plus mRNA, ×-×; plus mRNA plus 2 µl extract of strain MT200D (mutant), ●-●; plus mRNA plus 0.5 µl extract of strain MT106B (wild type), □-□; plus mRNA plus 2 µl extract of strain MT106B (wild type), ■-■. (**C**) Translation in extract MT200D (mutant). Incubation temperature was 20°C. No mRNA added, ○-○; plus mRNA, ×-×; plus mRNA plus 0.2 µl mammalian eIF-4B, ●-●; plus mRNA plus 0.6 µl mammalian eIF-4B, □-□; plus mRNA plus 2 µl mammalian eIF-4B, ■-■. (**D**) Translation in extract MT106B (wild type). Incubation temperature was 20°C. No mRNA added, ○-○; plus mRNA, ×-×; plus mRNA plus 2 µl extract of strain MT200D (mutant), ●-●; plus mRNA plus 2 µl extract of strain MT106B (wild-type), ■-■.

not shown). The defect in translation at 20°C in the mutant extract can be corrected by addition of small amounts of wild-type extract (Figure 4B) or mammalian eIF-4B overexpressed in *E.coli* (Figure 4C), but not by the addition of mutant extract which does not contain Tif3 (Figure 4B) or a truncated version of eIF-4B (amino acid 1−312; data not shown). The roughly 2-fold stimulation obtained brings the activity of the mutant extract back to the level of the wild-type extract. Wild-type extract is not stimulated by either mutant or wild-type extract. Rather, a slight inhibition was often but not always observed (Figure 4D). Presumably, the only difference between mutant and wild-type extract is the presence of Tif3 in the latter. Therefore, the *in vitro* translation data suggest that Tif3 is a translation factor.

Tif3 shows helicase activity in vivo

Definitive proof for a role of Tif3 in translation requires demonstration of translation factor activity *in vivo*. Specifically, we were interested in a possible role in unwinding of RNA secondary structure in the leader region of mRNA. To address this point, plasmid constructs encoding β-galactosidase mRNA with RNA secondary structure of increasing stability in their leader regions were transformed into the yeast strain RCB1 (*TIF3* gene deleted; Table II) in the presence or absence of the *TIF3* gene on a centromeric plasmid under the *GAL1* promoter (Materials and methods). These cells were incubated in galactose-containing medium and the synthesis of β-galactosidase from different mRNAs was measured (Figure 5). Translation of mRNA with little secondary structure (Figure 5A, construct p281; −8.7 kcal/mol) is 1.5- to 2-fold stimulated by Tif3 at 30°C. Lowering the temperature to 25 or 22.5°C results in slightly higher (2- to 4-fold) stimulation. At 37°C the translation of this mRNA is not stimulated but 2-fold inhibited by the expression of the *TIF3* gene. Introduction of more stable RNA secondary structure into the leader of the β-galactosidase mRNA (Figure 5B, construct p281-2; −14.1 kcal/mol) inhibits translation and enhances the dependence on Tif3 for translation. Tif3 stimulates translation of this mRNA 2- to 3-fold at 37°C, 5- to 7-fold at 30°C and 5- to 15-fold at 25°C or 22.5°C. An mRNA with even more stable RNA secondary structure in the leader

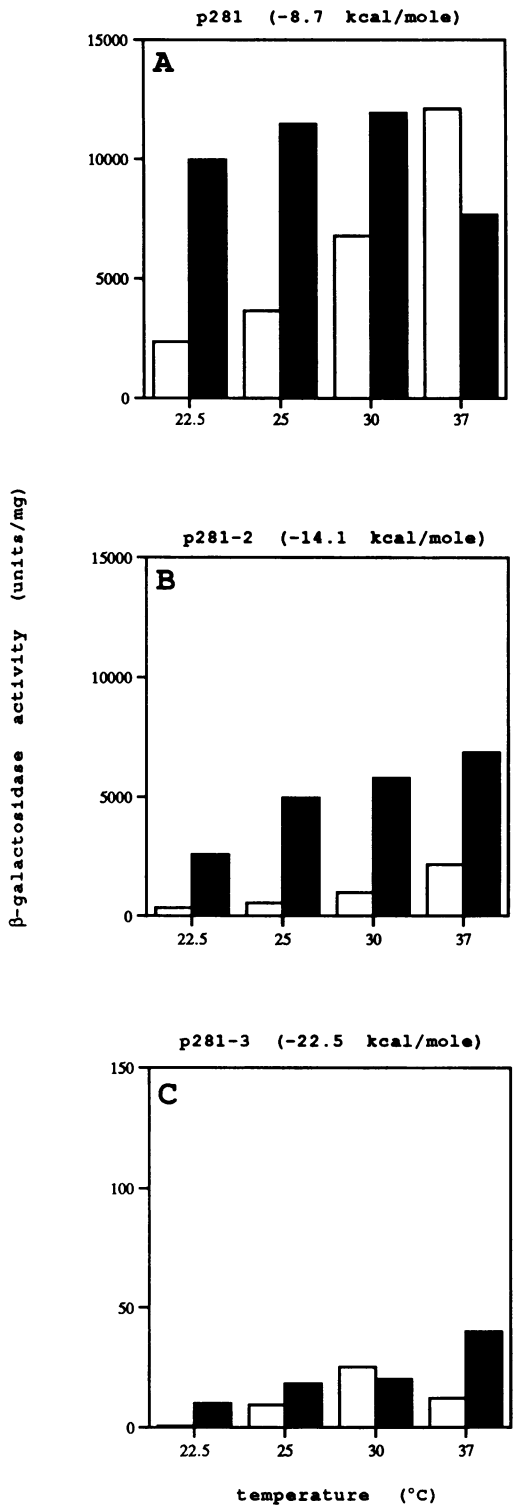

Fig. 5. *In vivo* translation of β-galactosidase mRNA at different temperatures. β-galactosidase activity (in units/mg) expressed from cells harbouring p281, p281-2 or p281-3 is presented. Open bars correspond to *TIF3* gene-disrupted cells (strain RCB1) carrying the control vector p301-HIS, black bars correspond to *TIF3* gene-disrupted cells (strain RCB1) expressing Tif3 from the plasmid p301-HIS3/GAL−TIF3. The data represent a typical experiment (all experiments were done three times).

region (Figure 5C, construct p281-3; −22.5 kcal/mol) was very poorly translated under all conditions (translational activity is reduced by a factor of 100- to 200-fold compared with the constructs with lower secondary structure). This result is consistent with previous findings (Baim and Sherman, 1988; Cigan *et al.*, 1988), indicating that secondary structure in the leader region of mRNA of more than −20 kcal/mol is strongly inhibitory for translation in *S.cerevisiae*. Northern blot analysis of total RNA extracted from the different yeast cells incubated at different temperatures showed that the β-galactosidase mRNA levels were equally high for cells harbouring the p281 and p281-2 constructs but 4- to 5-fold lower for the p281-3 construct (results not shown). We do not know if the reduced level of β-galactosidase mRNA carrying the higher secondary structure at the 5′-end is due to a lower transcriptional rate or a higher mRNA turnover. Nevertheless, the reduced level of mRNA cannot account for the drastic reduction in translational activity.

The finding that Tif3 stimulates translation *in vivo* and that the degree of stimulation increases with lower incubation temperature and higher stability of RNA secondary structure in the leader of the mRNA suggests that Tif3 is involved in RNA secondary structure melting during translation.

Discussion

We believe that the protein Tif3 is the *S.cerevisiae* homologue of mammalian translation initiation factor eIF-4B. The following structural and functional properties of Tif3 support this claim. (i) Tif3 shows significant similarity to eIF-4B at the amino acid level along the entire sequence. It shares with eIF-4B an RNA binding domain (appropriately spaced RNP1 and RNP2 elements) in the N-terminal half followed by repeated acidic amino acid sequence motifs. (ii) Addition of crude extract containing Tif3 to an extract derived from a *TIF3* gene-disrupted strain stimulates translation of total yeast RNA, while addition of crude extract lacking Tif3 does not. Mammalian eIF-4B (purified from overexpressing *E.coli* cells) was as active as crude Tif3-containing yeast fractions in this system. (iii) Tif3 stimulates translation of β-galactosidase reporter mRNA *in vivo*. Since transcription of the reporter gene is not influenced by Tif3 *in vivo*, we believe that it affects translation directly. (iv) The degree of translational stimulation of the reporter mRNA *in vivo* depends on the stability of RNA secondary structure in the 5′ leader sequence. Either lower incubation temperature or introduction of stable secondary structure in the 5′-leader region of the reporter mRNA leads to a higher dependence on Tif3 for translation. This indicates that Tif3 is an RNA helicase or part of an RNA helicase. Such a function was assigned to mammalian eIF-4B based on *in vitro* helicase assays in combination with eIF-4A (Rozen *et al.*, 1990). (v) Finally, there is genetic evidence for an interaction of Tif3 and eIF-4A in yeast: overexpression of Tif3 in an *S.cerevisiae* strain expressing a temperature-sensitive eIF-4A suppresses the conditional lethal phenotype (Coppolecchia *et al.*, accompanying paper). The ability to interact with eIF-4A is expected since eIF-4B strongly stimulates RNA-dependent ATPase activity of eIF-4A (Merrick, 1992) and forms *in vitro* an active RNA helicase with eIF-4A (Rozen *et al.*, 1990).

Translation initiation factors of *S.cerevisiae* involved in mRNA binding and RNA secondary structure unwinding such as eIF-4A (Linder and Slonimski, 1989), eIF-4E (Altmann *et al.*, 1987), the largest subunit of eIF-4F (Goyer

et al., 1993), Ssl1 (Yoon *et al.*, 1992) and Ssl2 (Gulyas and Donahue, 1992) are essential for growth. It was therefore rather surprising to find that the *TIF3* gene is not essential. We do not have a final explanation for this finding but consider the following possibilities. (i) A second *TIF3* gene is present in the genome of the yeast. Duplicate genes encoding an initiation factor are not uncommon in *S.cerevisiae*. The factors eIF-4A (Linder and Slonimski, 1989), eIF-4D (Schnier *et al.*, 1991) and the largest subunit of eIF-4F (Goyer *et al.*, 1993) are all encoded by two genes. If a second gene for Tif3 exists, it is not very conserved at the nucleotide sequence level since Southern hybridization under stringent conditions showed no evidence for a second gene. (ii) Other proteins can partially substitute for Tif3. Candidates are Ssl1 and Ssl2 or eIF-4F. However, eIF-4F cannot substitute for eIF-4B *in vitro* to form an active RNA helicase with eIF-4A (Rozen *et al.*, 1990). (iii) Tif3 is a regulatory subunit of an RNA helicase complex. Modulation of Tif3 activity preferentially influences translation of mRNA with strong secondary structure in the leader region. Little is known about regulation of translation at the level of mRNA secondary structure unwinding. Support for this type of regulation comes from the observation that *X.laevis* oocytes are able for a short period after fertilization to translate a reporter mRNA with a strong secondary structure in the 5′ leader region (Fu *et al.*, 1991). Regulation of Tif3 activity could be achieved by phosphorylation. Mammalian eIF-4B is a phosphoprotein and increased phosphorylation of eIF-4B correlates with enhanced translational activity in mammalian cells under a variety of physiological conditions (Hershey, 1991).

The cloning of the *TIF3* gene described in this report is the first step towards elucidating the precise role of this factor in translation in *S.cerevisiae* as well as in higher eukaryotes.

Materials and methods

S.cerevisiae strains

The genotypes of yeast strains used in this report are shown in Table II.

Manipulation of yeast cells

Yeast cells were transformed using the lithium acetate method (Ito *et al.*, 1983). Other methods were according to established procedures (Sherman *et al.*, 1986).

DNA manipulations

Unless indicated otherwise DNA manipulations were done as described by Sambrook *et al.* (1989).

Disruption of the TIF3 gene

A 123 bp genomic DNA fragment encoding part of the *TIF3* gene (see Results) was cut out from the plasmid pEX (Stanley and Luzio, 1984) as an *Eco*RI−*Sal*I restriction fragment and inserted into the plasmid pYip5 (plasmid pBR322 carrying the *S.cerevisiae URA3* gene). The resulting plasmid was termed pB236; it was linearized at the unique *Mlu*I site in the 123 bp genomic DNA fragment (indicated in Figure 1), transformed into the diploid *S.cerevisiae* strain X246 and Ura$^+$ transformants selected. Tetrad analysis after sporulation of Ura$^+$ diploids revealed two large Ura$^-$ and two small Ura$^+$ colonies per tetrad dissected. One segregant, MT200D, was further analysed: Southern blot analysis showed that pB236 was integrated at a single site and disrupted the *TIF3* gene. Northern blot analysis confirmed the synthesis of an enlarged *TIF3* mRNA in these cells (Results; Figure 3).

Cloning of the TIF3 gene

Genomic DNA of strain MT200D was digested either with the restriction enzyme *Sal*I or *Eco*RI and the resulting DNA fragments ligated (self-ligation). Transformation of the ligation products into *E.coli* and selection for ampicillin-resistant cells resulted in the isolation of clones carrying plasmid pB236 containing the first part of the *TIF3* gene (plasmid p130R, *Eco*RI digestion) or the second part (plasmid p130S, *Sal*I digestion). The *TIF3* *Eco*RI−*Sal*I fragment was cut out from plasmid p130R and inserted into *Eco*RI/*Sal*I-cut plasmid pUC9. An *Eco*RI DNA fragment carrying *TRP1/ARS1* was then inserted into the *Eco*RI site of this pUC9 construct to give plasmid pB372. Finally, the *Mlu*I−*Sal*I DNA fragment from plasmid p130S was inserted into *Mlu*I/*Sal*I-cut plasmid pB372 to recombine the first and second half of the *TIF3* gene. This plasmid was termed pB382.

Table II. Yeast strains used in this study

Strain	Phenotype	Reference
MT106B	*MATa ura3 leu2 trp1*	This study
X246	*MATa/α ura3/ura3 his3/HIS3 leu2/LEU2 trp1/TRP1*	This study
MT200D	*MATα tif3::URA3 ura3 leu2 trp1*	This study
CWO4[a]	*MATa ade2 his3 leu2 trp1 ura3 can^R*	Coppolecchia *et al.* (1993)
RCB1[a]	*MATa tif3::ADE2 ade2 his3 leu2 trp1 ura3 can^R*	Coppolecchia *et al.* (1993)

[a]Obtained from Patrick Linder, Biocenter, Basel, Switzerland.

DNA sequencing

A 4.6 kb *Eco*RI DNA fragment carrying the *TIF3* gene was cut out from plasmid pB382 and inserted into the vector pRS314 (Sikorski and Hieter, 1989). This construct was transformed into *E.coli* and single-stranded DNA prepared by infection of transformed cells with helper phage (Stratagene). Single-stranded DNA was sequenced (Sanger *et al.*, 1977) with synthetic deoxy-oligonucleotide primers using the Sequenase kit (US Biochemicals, Cleveland, OH). DNA sequence comparison with sequences in the GENEMBL data bank were done with the FastA program (Pearson and Lipman, 1988). The sequence of the *TIF3* gene was submitted to the EMBL databank (accession number X71996).

Southern blot analysis

Genomic DNA was prepared from wild-type strain MT106B. Aliquots of ~10 μg of DNA were cut with restriction enzymes and fractionated on a 0.7% agarose gel. DNA fragments were transferred to nitrocellulose (GeneScreen, NEN) by capillary transfer and hybridized to an α-^{32}P-labelled RNA probe (6×10^7 c.p.m.). The RNA probe was synthesized with T7 RNA polymerase by *in vitro* transcription of the plasmid Bluescript (Promega) carrying the *TIF3 Xho*I DNA fragment. The plasmid was linearized downstream of the insert by digestion with *Sal*I. Hybridization was carried out in 6 × SSC, 5 × Denhardt's solution, 0.5% SDS, 100 μg/ml herring sperm DNA for 16 h at 68°C. The blots were washed for 60 min in 0.1 × SSC, 0.5% SDS at 37°C and exposed to Fuji X-ray film for 66 h at −70°C with intensifying screen.

Northern blot analysis

Total RNA from the strains MT106B and MT200D was prepared as described (Altmann *et al.*, 1985). About 12 μg of RNA were fractionated on a 0.8% formaldehyde−agarose gel (Sambrook *et al.*, 1989), transferred to nitrocellulose, hybridized with a labelled RNA probe (see Southern blot analysis, above) and washed as described above for Southern blot analysis. An additional washing step in 0.1 × SSC, 0.5% SDS for 15 min at 68°C was done. Exposure to Fuji X-ray film was for 16 h with intensifying screen.

In vitro translation

Extracts from the yeast strains MT106B and MT200D were prepared and cell-free translation performed as described by Altmann *et al.* (1985).

In vivo translation of β-galactosidase mRNA.

β-Galactosidase constructs with secondary structure. The symmetric deoxynucleotide 5′-GATCCTAG-3′ was ligated into the unique *Bgl*II site of plasmid p281 (the restriction site is located 22 bases downstream of the transcription initiation site of the β-galactosidase open reading frame under the control of the *GAL1* promoter; Müller *et al.*, 1987). Several clones obtained were analysed by sequencing. For this study the derivatives p281-2 carrying two tandem repeats and p281-3 carrying three tandem repeats of the octanucleotide were selected.

The predicted secondary structure for the 5'-leader region of *lacZ* transcripts (program FOLD of the GCG software package; method of Zucker) corresponds to −8.7 kcal/mol for p281, −14.1 kcal/mol for p281-2 and −22.5 kcal/mol for p281-3.

TIF3 under the control of the GAL1 promoter. Using PCR amplification a *TIF3* gene version was engineered in which the entire 5' leader region had been replaced by the synthetic sequence 5'- C CGG ATC CTG AAA GGA AGA AAA *ATG* (ATG translation initiation codon italicized) and subcloned into the polylinker of the vector p301-HIS3, a centromeric yeast shuttle vector. This gave the construct p301-HIS3/GAL−TIF3 which expresses the *TIF3* gene from the galactose-regulated *GAL1* promoter.

Expression of β-galactosidase in a Tif3-deficient yeast strain. The plasmids p301-HIS3/GAL-TIF3 and p301-HIS3 (as negative control) were transformed together with the p281 derivative plasmids into the *TIF3* gene-disrupted haploid strain RCB1.

Transformants carrying either p281, p281-2 or p281-3 together with p301-HIS3/GAL−TIF3 or p301-HIS3 were grown to confluency (100 ml culture) in 0.67% YNB (yeast nitrogen base; supplemented with tryptophan and leucine) and 2% glucose at 30°C. Cells were washed once with distilled water, resuspended in fresh minimal medium containing 2% galactose (instead of glucose) and incubated at the desired temperature for 20 h. Cell extracts were prepared and β-galactosidase activity determined (Müller *et al.*, 1987) or RNA isolated (Altmann *et al.*, 1985).

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