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The *Saccharomyces cerevisiae* translation initiation factor Tif3 and its mammalian homologue, eIF-4B, have RNA annealing activity

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The *Saccharomyces cerevisiae TIF3* gene encodes the yeast homologue of mammalian translation initiation factor eIF-4B. We have added six histidine residues to the C-terminus of Tif3 protein (Tif3–His6p) and purified the tagged protein by affinity chromatography. Tif3–His6p stimulates translation and mRNA binding to ribosomes in a Tif3-dependent *in vitro* system. Furthermore, it binds to single-stranded RNA and catalyses the annealing of partially complementary RNA strands *in vitro*. In parallel experiments, RNA annealing activity could also be demonstrated for mammalian eIF-4B. A role for Tif3/eIF-4B and RNA annealing activity in the scanning process is proposed.

Keywords: RNA annealing/RNA binding protein/*Saccharomyces cerevisiae*/translation initiation factor

Introduction

RNA interactions play a fundamental role in initiation of protein synthesis. In eukaryotes, initiation of translation involves: (i) recognition of the ^{7}mGpppX cap structure at the 5′ end of mRNA by the cap binding protein eIF-4E and associated factors (eIF-4F); (ii) formation of a 40S·eIF-2·GTP·Met-tRNA$_i$ complex; (iii) binding of this complex to or close to the 5′ end of the mRNA and movement of the 40S subunit with attached initiation factors and Met-tRNA$_i$ on the mRNA in the 5′→3′ direction (scanning) and (iv) recognition of the initiator AUG codon by the 40S subunit and joining of the 60S subunit to form 80S initiation complexes. Initiation factors eIF-4A and eIF-4B and the hydrolysis of ATP are required for binding of ribosomes to mRNA and/or scanning. For recent reviews of translation initiation, see Kozak (1989), Hershey (1991), Merrick (1992), Altmann and Trachsel (1993), Rhoads (1993) and Sonenberg (1993).

Recently, we and others have cloned the *TIF3* gene (also called *STM1*) encoding the *Saccharomyces cerevisiae* homologue of mammalian eIF-4B (Altmann *et al.*, 1993; Coppolecchia *et al.*, 1993). *TIF3* carries an open reading frame (ORF) encoding a protein of 436 amino acids with a calculated molecular weight of 48.5 kDa. Alignment of Tif3 protein (Tif3p) and mammalian eIF-4B amino acid sequences reveals 26% identity (115 perfect amino acid matches) and 50% similarity evenly distributed along the protein sequence, except for the 45 C-terminal amino acids of eIF-4B (total length 611 amino acids) which are missing in Tif3p (Altmann *et al.*, 1993). The function of this C-terminal segment of eIF-4B is not known.

The amino acid sequences of Tif3p and eIF-4B (Milburn *et al.*, 1990) suggest that these proteins bind to RNA. Both proteins contain in their N-terminal region an RNA recognition motif (RRM) which consists of the RNP2 and RNP1 sequence motifs which are part of RNA binding sites in many proteins (Kenan *et al.*, 1991). In Tif3p, the RNP2 motif (amino acids 103–108) and RNP1 motif (aa 147–154) are followed by a 7-fold repeat of 26 amino acids (aa 183–352) rich in serines, arginines and acidic amino acids which could be involved in RNA interactions (Altmann *et al.*, 1993). The RRM in eIF-4B (located between amino acids 97 and 175) is followed by a short several-fold repeat of the amino acid sequence DRYG (aa 214–327). Recent functional analysis of eIF-4B deletion mutants has shown that the RRM motif in eIF-4B is not sufficient for RNA binding and that the C-terminal region of the protein containing a cluster of basic amino acids (located between amino acids 367 and 423) is essential for RNA binding and for eIF-4A-dependent unwinding of RNA duplex structures (Méthot *et al.*, 1994; Naranda *et al.*, 1994). The contribution to RNA binding activity of the RRM motif and the C-terminal sequences rich in basic amino acids of Tif3p and eIF-4B are not known at present (see also Discussion).

Disruption of the *TIF3* gene in *S.cerevisiae* results in a slow growth, cold- and temperature-sensitive phenotype (see Figure 1). Though the existence of further *TIF3*-related genes cannot be excluded, attempts to clone them by DNA hybridization have so far been unsuccesful (Altmann *et al.*, 1993; Coppolecchia *et al.*, 1993). Tif3p plays an important role in initiation of translation: it was shown that *in vivo* reporter mRNAs with increasing secondary structure at their 5′-untranslated region (UTR) are less efficiently translated in the absence of the *TIF3* gene, indicating a role in the unwinding of mRNA structures by 40S ribosomes (Altmann *et al.*, 1993). Further evidence for the involvement of Tif3 protein in this process is provided by the finding that overexpression of *TIF3 in vivo* suppresses a temperature-sensitive mutation in Tif1p, the yeast homologue of eIF-4A, an ATP-dependent RNA helicase involved in initiation of protein synthesis (Coppolecchia *et al.*, 1993).

Here, we analyse RNA interactions catalysed by Tif3p. A new RNA annealing activity which is ATP- and Mg^{2+}-independent is described for Tif3p and mammalian eIF-4B.

	Temperature		
	20°C	30°C	37°C
-TIF3	7.0 h	4.0 h	15.0 h
+TIF3-His6p	3.7 h	2.9 h	3.3 h

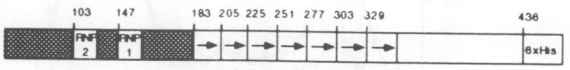

Fig. 1. Growth properties of yeast with the chromosomal *TIF3* gene deleted and expressing Tif3–His6p. The cell doubling time of strain *RCB-1C* transformed with p301-HIS3/GAL-TIF3-His6 (or p301-HIS3 as control) cultivated in liquid YP medium containing 2% galactose at different temperatures is presented. *–TIF3* corresponds to cells transformed only with the vector pGAL301HIS3 used for the construct *TIF3–His6* (see Materials and methods). A scheme with characteristic domains of Tif3–His6p is presented: the RNP2 consensus sequence at amino acid position 103, the RNP1 at amino acid 147, the 7-fold 26 amino acid repeat at positions 183, 205, 225, 251, 277, 303 and 329 (boxes with arrows) and the His tag starting after position 436 at the C-terminus of Tif3.

Results

Purification and characterization of Tif3p derivatives

To further characterize Tif3p, we expressed the whole *TIF3* ORF (aa 1–436) with a tail of six histidine residues at the C-terminus (see scheme in Figure 1) from a plasmid under the control of the regulated GAL1/10 promoter in the *TIF3*-disrupted strain *RCB-1C* (Coppolecchia *et al.*, 1993). *TIF3–His6* was able to complement the *TIF3* null allele mutation, as judged from its ability to correct the cold- and temperature-sensitive phenotype of strain *RCB-1C* on galactose-containing media (Altmann *et al.*, 1993; Coppolecchia *et al.*, 1993). Transformation of the *TIF3*-deleted haploid strain with a plasmid carrying the *TIF3–His6* gene reduced the generation time from 7 h to 3.7 h at 20°C on galactose-containing media (Figure 1). The effect was even more drastic at 37°C: the doubling time was reduced from 15 h to 3.3 h. No significant differences in growth were observed among *RCB-1C* derivatives transformed with different *TIF3* constructs when cells were grown on glucose-containing media, confirming that galactose-dependent Tif3 expression had cured the cold- and temperature-sensitive phenotype of the *TIF3* null allele strain (not shown). This indicates that the attached C-terminal His6× tag did not affect the activity of Tif3p.

Tif3–His6p was purified from yeast cells cultivated in galactose-containing media by affinity chromatography on a Ni^{2+} column and the identity of the purified protein verified by Western blotting and reaction with an anti-Tif3 antibody (Figure 2A). A major polypeptide band with the expected molecular weight of ~48 kDa (Tif3–His6p; lanes 3, 4 and 6) and minor shorter polypeptides could be detected. The latter appear to be Tif3p degradation products, as judged from their reaction with the polyclonal antibody against Tif3p (Figure 2A, lane 6).

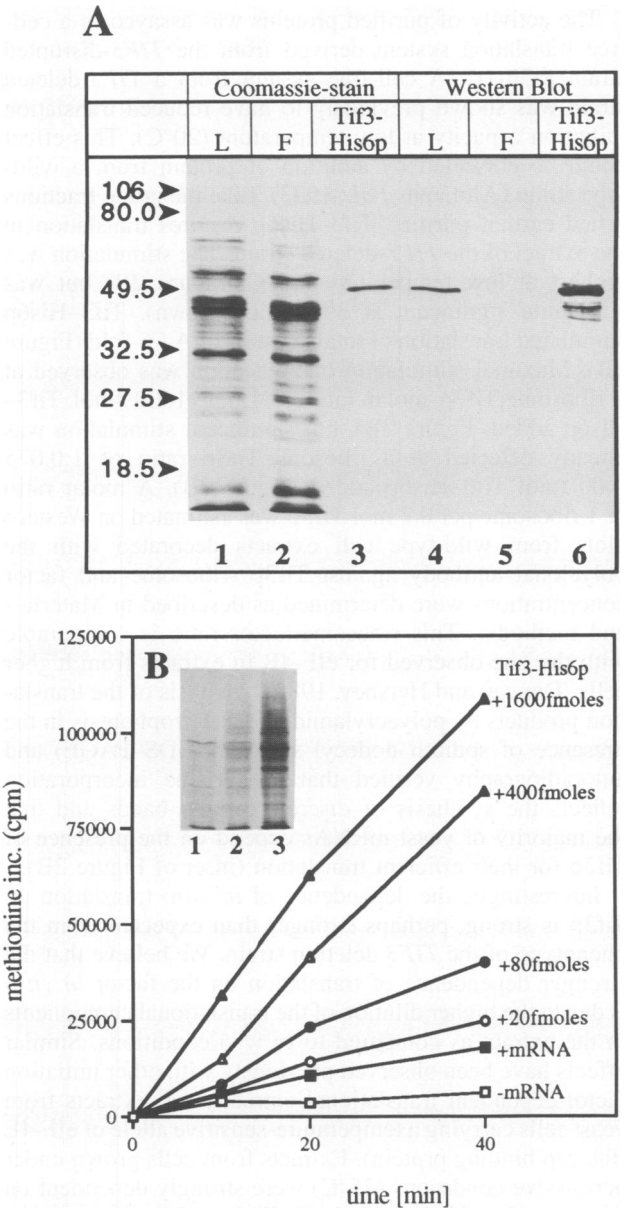

Fig. 2. Characterization of Tif3–His6p. (**A**) SDS–PAGE analysis: Coomassie Blue R-250 staining (lanes 1–3) and Western blot (lanes 4–6). Lane 1, ~50 µg of total yeast extract of strain *RCB-1C* expressing Tif3–His6p (L, load); lane 2, the same amount of protein after passage through a Ni^{2+} column (F, flow); lane 3, ~50 ng protein eluted from the Ni^{2+} column and further purified on a DEAE column. Lanes 4–6 are identical to lanes 1–3 but protein was transferred to nitrocellulose and reacted with polyclonal rat anti-Tif3p antibody at a dilution of 1:1000 (in 0.5% bovine serum albumin). The peroxidase-stained blot is shown. (**B**) *In vitro* translation in extracts derived from the *TIF3*-disrupted strain *RCB-1C*. Translation was done at 20°C in 12 µl incubation mixtures programmed with 5 µg of total yeast RNA as mRNA. Aliquots (3 µl) were removed at the times indicated and [^{35}S]methionine incorporation measured. □–□, no mRNA added; ■–■, plus mRNA, minus Tif3–His6p; ○–○ , plus mRNA, plus 20 fmol Tif3–His6p; ●–●, plus mRNA, plus 80 fmol Tif3–His6p; △–△, plus mRNA, plus 400 fmol Tif3–His6p; ▲–▲, plus mRNA, plus 1600 fmol Tif3–His6p. **Inset:** 3 µl aliquots (after incubation for 40 min) were fractionated by SDS–PAGE and the gels prepared for autoradiography. The autoradiograms are shown. Lane 1, no mRNA added; lane 2, plus mRNA, minus Tif3p; lane 3, plus 1600 fmol Tif3–His6p.

The activity of purified proteins was assayed in a cell-free translation system derived from the *TIF3*-disrupted strain *RCB-1C*. A cell-free system from a *TIF3*-deleted strain was shown previously to have reduced translation initiation capacity at low temperature (20°C). This effect could be corrected by addition of protein from a wild-type strain (Altmann *et al.*, 1993). Like the crude fractions tested earlier, purified Tif3–His6p restores translation in the extract of the *TIF3*-deleted strain. The stimulation was highest at low temperature (20°C; Figure 2B) but was still quite significant at 30°C (not shown). Tif3–His6p stimulated translation of total yeast mRNA ~6-fold (Figure 2B). Maximal stimulation of translation was observed at a ribosome:Tif3p molar ratio of 1:0.3 (1600 fmol Tif3–His6p added; Figure 2B), but significant stimulation was already detected at a ribosome:Tif3p ratio of 1:0.075 (400 fmol Tif3–His6p added; Figure 2B). A molar ratio of 1 ribosome per 0.2 mol Tif3p was estimated on Western blots from wild-type cell extracts decorated with the polyclonal antibody against Tif3p (ribosome and factor concentrations were determined as described in Materials and methods). This ribosome:factor ratio is comparable with the one observed for eIF-4B in extracts from higher cells (Duncan and Hershey, 1983). Analysis of the translation products by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS–PAGE) and autoradiography verified that methionine incorporation reflects the synthesis of discrete protein bands and that the majority of yeast mRNAs depend on the presence of Tif3p for their efficient translation (inset of Figure 2B).

Interestingly, the dependence of *in vitro* translation on Tif3p is strong, perhaps stronger than expected from the phenotype of the *TIF3* deletion strain. We believe that the stronger dependence of translation on the factor *in vitro* is due to the higher dilution of the translational components in the extract as compared to *in vivo* conditions. Similar effects have been observed previously with other initiation factor-dependent translational extracts, e.g. extracts from yeast cells carrying a temperature-sensitive allele of eIF-4E (the cap binding protein). Extracts from cells grown under permissive conditions (25°C) were strongly dependent on exogenously added purified eIF-4E for efficient translation (Altmann *et al.*, 1989).

Tif3p stimulates mRNA binding to ribosomes and binds to RNA

Which steps in translation initiation are catalysed by Tif3p? Human eIF-4B is known to be involved in mRNA binding to ribosomes (Hershey, 1991; Rhoads, 1991). To test whether this is also true for Tif3p, we measured binding of a 166 nucleotide long labelled capped mRNA fragment carrying the 5′-UTR (55 nucleotides), the AUG initiation codon and the first 36 codons of CAT mRNA to ribosomes in the cell-free system derived from the *TIF3*-disrupted strain *RCB-1C* (Figure 3). In the presence of Tif3–His6p, a significant mRNA fraction was bound to 80S ribosomes, and to a lesser extent to 40S ribosomes, after 3 min incubation at 20°C (Figure 3, closed circles). Omission of Tif3–His6p resulted in a 4- to 5-fold reduction of mRNA associated with 80S, and a 2- to 3-fold reduction of mRNA associated with 40S ribosomes (Figure 3, open circles). Longer incubations did not change mRNA distribution patterns (not shown). This result demonstrates

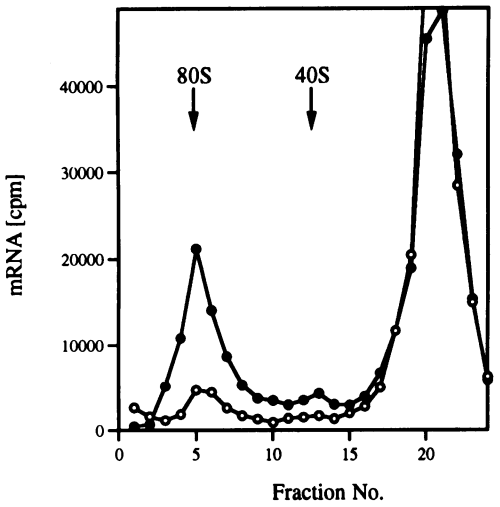

Fig. 3. Sucrose gradient fractionation of translation reactions. *RCB-1C* extract was incubated under translation conditions for 3 min at 20°C and binding of α-^{32}P-labelled mRNA to ribosomes was analysed by ultracentrifugation, gradient fractionation and liquid scintillation counting (for details, see Materials and methods). Incubation conditions: ●–●, plus 4 pmol Tif3–His6p; ○–○, minus Tif3p. Arrows indicate the positions of 80S and 40S ribosomes in the gradient. Sedimentation was from right to left.

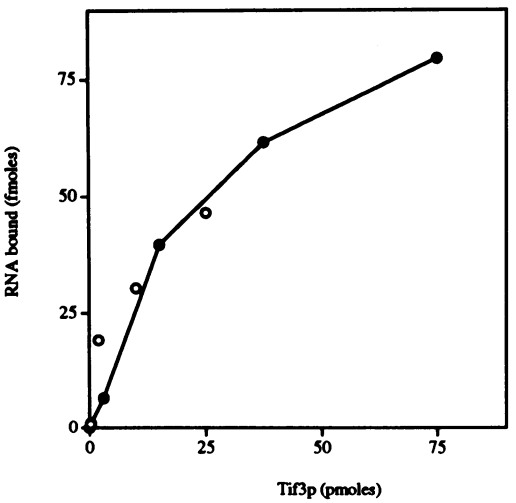

Fig. 4. RNA binding activity of Tif3–His6p. Nitrocellulose filter binding assays were done with 450 fmol of a 41 nucleotide long α-^{32}P-labelled single-stranded RNA and increasing amounts of Tif3–His6p. White and black dots represent the results from two different experiments.

that the yeast homologue of eIF-4B promotes, directly or indirectly, the binding of mRNA to the 40S·eIF2·GTP·Met-tRNA$_i$ pre-initiation complex.

Both Tif3p and mammalian eIF-4B carry RNA recognition motifs in their N-terminal part, followed by serine, arginine and acidic amino acid rich repeats which could contribute to RNA binding. In the case of eIF-4B, a region containing a cluster of basic amino acids has been shown to be essential for RNA binding (see Introduction). We therefore tested Tif3–His6p for its ability to bind RNA by incubating the protein with capped and uncapped (*in vitro* transcribed) RNA and filtrating the RNA–protein complexes through a nitrocellulose filter (Figure 4).

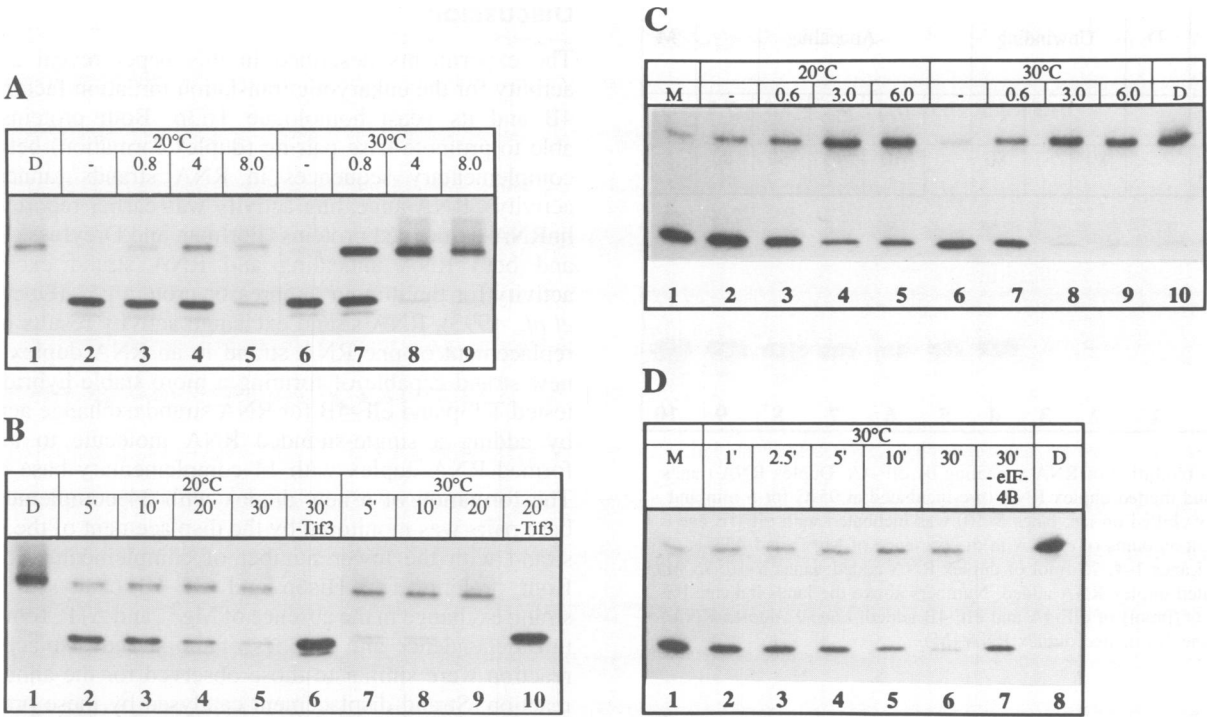

Fig. 5. RNA strand annealing with Tif3–His6p and mammalian eIF-4B. (**A**) Titration of Tif3–His6p at different temperatures. 75 fmol of melted duplex RNA (except lane 1) was incubated for 20 min with increasing amounts of Tif3–His6p at 20°C or 30°C. Lane 1, duplex RNA (D); lanes 2 and 6, no Tif3–His6p added; lanes 3 and 7, + 0.8 pmol Tif3–His6p; lanes 4 and 8, + 4 pmol Tif3–His6p; lanes 5 and 9, + 8 pmol Tif3–His6p. (**B**) Kinetics of RNA annealing with Tif3–His6p at different temperatures. 75 fmol of melted duplex RNA (except lane 1) was incubated with Tif3–His6p (except lanes 1, 6 and 10) at 20°C or 30°C for the indicated times. Lane 1, duplex RNA (D); lanes 2–5 and 7–10, incubations in the presence of 4 pmol Tif3–His6p; lanes 6 and 10, incubations in the absence of Tif3–His6p. (**C**) Titration of mammalian eIF-4B at different temperatures. 75 fmol of melted duplex RNA (except lane 10) was incubated for 20 min with different amounts of eIF-4B at 20°C or 30°C. Lane 1; melted duplex RNA (M); lanes 2 and 6, no eIF-4B added; lanes 3 and 7, + 0.6 pmol eIF-4B; lanes 4 and 8, + 3 pmol eIF-4B; lanes 5 and 9, + 6 pmol eIF-4B; lane 10, duplex RNA (D). (**D**) Kinetics of RNA annealing with mammalian eIF-4B at 30°C. 75 fmol of melted duplex RNA (except lane 8) was incubated with eIF-4B (except lanes 1, 7 and 8) at 30°C for the indicated times. Lane 1, melted duplex RNA (M); lanes 2–6, incubations in the presence of 3 pmol eIF-4B; lane 7, incubations in the absence of eIF-4B; lane 8, duplex RNA (D).

Tif3–His6p binds RNA in this type of experiment; no significant difference in the binding of capped and uncapped RNA was found (not shown).

Tif3p and eIF-4B anneal complementary RNA strands

Several nuclear RNA binding proteins involved in RNA processing catalyse RNA duplex formation (annealing) between RNAs with complementary sequences (Portman and Dreyfuss, 1994). Therefore, we next addressed the question whether cytoplasmic RNA binding proteins involved in initiation of translation can catalyse RNA annealing. Indeed, incubation of partially complementary RNA strands with increasing amounts of Tif3–His6p leads to their annealing to form a heteroduplex (Figure 5). The reaction does not require ATP or Mg^{2+} and is temperature dependent, with higher activity observed at 30°C (Figure 5A and B). Similar annealing activity was observed at 37°C (not shown). Very little spontaneous self-annealing of RNA strands was observed in the absence of the protein (Figure 5A, lanes 2 and 6; B, lanes 6 and 10). At 4°C no RNA duplex formation was observed, even in the presence of high Tif3p concentrations (not shown). Maximal annealing of duplex RNA (total length of 109 nucleotides; see Material and methods) was observed at protein concentrations of 400–800 nM, which corresponds to a protein:nucleotide ratio of between 1:2 and 1:1 or to a protein:RNA duplex ratio of between 50:1 and 100:1 (4–8 pmol factor per 75 fmol RNA duplex; Figure 5A, lanes 8 and 9). Similar *in vitro* annealing activities have been described for the hnRNP proteins A1 and C1, while hnRNP protein U showed a maximum annealing activity at a protein:nucleotide ratio of 1:16 (Portman and Dreyfuss, 1994). The annealing reaction catalysed by Tif3p is time dependent (Figure 5B). At 20°C, >50% of the duplex was annealed after 20 min (Figure 5B, lane 4) and at 30°C, >50% was already annealed after 5 min reaction time (Figure 5B, lane 7).

Like Tif3p, its human homologue eIF-4B is able to promote efficient RNA strand annealing (Figure 5C and D). ATP and/or Mg^{2+} were not required in the annealing reaction and maximal enzymatic activity was observed between 30°C (Figure 5C, lanes 6–9) and 37°C (not shown). Similar protein:nucleotide ratios to those for Tif3p were required for efficient annealing; 300–600 nM eIF-4B, corresponding to a protein:nucleotide ratio of 1:2.7–1:1.4, were required for 100% heteroduplex formation at 30°C (Figure 5C, lanes 8 and 9). However, the kinetics of annealing with eIF-4B was slower than with Tif3p (compare Figure 5D, lanes 2 –6 with B, lanes 7–9).

Note that the label in the annealed RNA band is sometimes smaller than the input label (melted duplex RNA). This is probably due to incomplete digestion of RNA–protein complexes (proteinase K; see Materials and

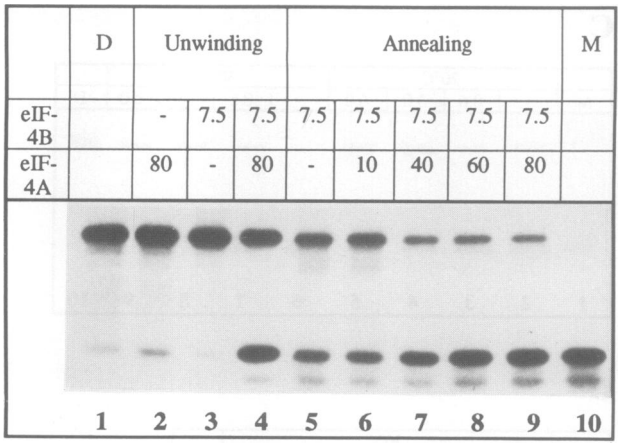

Fig. 6. Inhibition of RNA annealing by eIF-4A. Duplex RNA (lanes 1–4) and melted duplex RNA (pre-incubated at 95°C for 5 min and quickly chilled on ice; lanes 5–10) was incubated with eIF-4B and different amounts of eIF-4A in the presence of Mg^{2+} and ATP at 37°C. Lanes 1–4, 75 fmol of duplex RNA added; lanes 5–10, 75 fmol of melted duplex RNA added. Numbers above the lanes indicate the amounts (pmol) of eIF-4A and eIF-4B added. Lane 1, duplex RNA (D); lane 10, melted duplex RNA (M).

methods) which results in a smear above the annealed RNA duplex.

Inhibition of RNA annealing by eIF-4A

The RNA annealing activity of eIF-4B and its yeast homologue Tif3p is surprising in the light of previous findings that eIF-4B, together with eIF-4A, is required to melt RNA duplex structures *in vitro* (Rozen *et al.*, 1990; Méthot *et al.*, 1994). We asked, therefore, if both eIF-4B-catalysed reactions, i.e. RNA annealing and unwinding of RNA duplexes, represent opposite reactions which depend on the relative amount of eIF-4A present in the reaction with respect to eIF-4B.

We incubated duplex RNA with eIF-4A and/or eIF-4B in the presence of Mg^{2+} and ATP (Figure 6, lanes 2–4). As shown earlier, significant unwinding of duplex RNA was only visible in the presence of both factors (lane 4). In this reaction, ~60% of the duplex was melted when a 10-fold excess of eIF-4A over eIF-4B was added. Under conditions where ~50% of RNA monomers had formed a duplex by eIF-4B-catalysed RNA annealing (lane 5), increasing amounts of eIF-4A (up to 10-fold excess eIF-4A over eIF-4B) significantly inhibited the annealing reaction (lanes 6–9). These data show that eIF-4B is capable of annealing complementary RNA strands in the absence of eIF-4A, while it catalyses the opposite reaction in the presence of eIF-4A. Rather large amounts of eIF-4A are required in order to obtain efficient unwinding *in vitro*.

We could not detect any significant RNA annealing inhibition or RNA unwinding in the presence of Tif3p, $[Mg\text{-}ATP]^{2-}$ and yeast eIF-4A (not shown). This was true despite the fact that purified yeast eIF-4A had been tested and found to be very active in an eIF-4A-dependent cell-free extract (Blum *et al.*, 1989). As yeast eIF-4A has been shown previously to be active in unwinding of duplex RNA with mammalian eIF-4B (Blum *et al.*, 1992), we assume that further yeast initiation factors (missing in our assay) are required to catalyse this reaction.

Discussion

The experiments described in this paper reveal a new activity for the eukaryotic translation initiation factor eIF-4B and its yeast homologue Tif3p. Both proteins are able to promote base pairing (duplex formation) between complementary sequences in RNA strands (annealing activity). RNA annealing activity was earlier reported for hnRNA-associated proteins (Portman and Dreyfuss, 1994) and both RNA annealing and RNA strand exchange activity for the tumour suppressor protein p53 (Oberosler *et al.*, 1993). RNA strand exchange activity results in the replacement of one RNA strand in an RNA duplex by a new strand capable of forming a more stable hybrid. We tested Tif3p and eIF-4B for RNA strand exchange activity by adding a single-stranded RNA molecule to a pre-formed RNA duplex with 14 complementary base pairs. The formation of a new duplex with 34 complementary base pairs was monitored by the displacement of the RNA strand with the lower number of complementary bases. Both proteins Tif3–His6p and eIF-4B catalysed RNA strand exchange in the absence of Mg^{2+} and ATP. Temperature dependence and kinetics of the strand displacement reaction were similar to those observed for the annealing reaction. Strand displacement catalysed by these proteins only occurred when the added single-stranded RNA allowed the formation of a new duplex with a higher number of complementary base pairs than the old duplex (results not shown). RNA strand exchange activity is probably a consequence of RNA strand annealing, in that hybridization of a third strand to partial duplex RNA leads to a thermodynamically driven zipper reaction.

To our knowledge, eIF-4B and Tif3p are the first cytoplasmic proteins involved in protein synthesis for which RNA annealing and RNA strand exchange activity has been demonstrated.

Base pairing between RNA strands plays a fundamental role in transcription, splicing, translation and a variety of other biochemical pathways. It is still not known in most cases how RNAs acquire their biologically active structures *in vivo*, but it is becoming evident that some RNA–RNA interactions are catalysed by proteins. Two different mechanisms for RNA annealing by hnRNP proteins were proposed, a 'matchmaker' and a 'chaperone' mechanism (Portman and Dreyfuss, 1994). As a matchmaker, the protein binds RNA strands and brings them into close contact to allow base pairing. As a chaperone, the protein binds to RNAs and changes their secondary structures to facilitate base pairing. Both models can account for protein-mediated promotion of intra- and inter-strand base pairing of RNA molecules.

Comparison of the structures of proteins with RNA annealing activity reveals a modular structure with common motifs (Biamonti and Riva, 1994). These proteins usually contain one or several of the well characterized RNA recognition motifs consisting of two short (6–8 amino acids) submotifs RNP1 and RNP2 separated by ~70 amino acids. These motifs are often localized in the N-terminal part of the protein. There is evidence suggesting that they recognize specific RNA sequences. In their C-terminal part these RNA binding proteins carry additional motifs such as the glycine-rich RGG box or SR domains. These auxiliary domains contribute in some proteins to

RNA binding or protein–protein interactions (or both). It has been suggested that RNA binding and protein dimerization might be the mechanism by which some of these proteins promote RNA annealing (Biamonti and Riva, 1994).

RNA annealing could be regulated by covalent protein modifications. As an example, protein kinase A-mediated phosphorylation of the glycine-rich domain of hnRNP protein A1 leads to *in vitro* inhibition of its RNA annealing activity (Cobianchi *et al.*, 1993).

Like other proteins with RNA annealing activity, eIF-4B and Tif3p carry RRM motifs in their N-terminal part, and repeated sequence motifs rich in basic and acidic amino acids in their C-terminal part. Both parts of eIF-4B and Tif3p contribute to RNA binding and play a role in translation initiation: the C-terminal domain carries an RNA binding site (Méthot *et al.*, 1994; Naranda *et al.*, 1994) which was identified in binding assays with RNAs of variable length and sequence. Therefore, it may be a sequence-unspecific RNA binding site. The N-terminally located RRM is required for RNA duplex unwinding (Méthot *et al.*, 1994), for *in vitro* RNA binding and *in vivo* eIF-4B function (Naranda *et al.*, 1994). It is not known at present whether the RRM of eIF-4B binds to a specific RNA sequence.

Like eIF-4B, Tif3p contains an RNA binding site in its C-terminal domain: experiments with a Tif3 protein whose N-terminal 173 amino acids (including the RRM) were deleted shows residual activity in translation, RNA annealing and RNA strand exchange *in vitro*. This protein is also partially active in complementation of the cold- and temperature-sensitive phenotype of a *TIF3* deletion *in vivo* (our unpublished results). The RRM located in the N-terminal domain of Tif3 must play an important role in translation since it is required for suppression of a temperature-sensitive mutation in the eIF-4A gene in the yeast *S.cerevisiae* (Coppolecchia *et al.*, 1993). The mechanistic details of RNA annealing and RNA strand exchange reactions catalysed by eIF-4B and Tif3p are not known. There is evidence that eIF-4B forms homodimers. In a two-hybrid system (Chien *et al.*, 1991), transcription of a reporter gene was obtained when eIF-4B was fused to both the DNA activation and the transcription activation domain. Deletion of the DRYG-rich motif of eIF-4B abolished its capacity to form homodimers. As shown *in vitro* by chemical cross-linking, dimerization of eIF-4B did not require ATP (Méthot and Sonenberg, unpublished results).

We favour a 'matchmaker'-type mechanism for RNA annealing. We know that both proteins bind single-stranded RNA and we suggest that two RNA strands are brought together through dimerization of the protein. RNA strand exchange would be promoted by the same mechanism.

The experiment presented in Figure 6 demonstrates that eIF-4B catalyses both RNA annealing and RNA duplex melting. How can this protein simultaneously stimulate RNA duplex melting and RNA duplex formation? At present, we have no answer but can speculate: it is possible that monomeric eIF-4B binds to single-stranded RNA produced by eIF-4A during the RNA duplex melting process. Through binding to one of the strands, eIF-4B would inhibit the immediate renaturation of RNA and stabilize the single-stranded RNA. To anneal the RNA strands it may have to form dimers, a reaction which would be dependent on the concentration of RNA and protein. Therefore, depending on the conditions chosen, one may observe RNA duplex melting or RNA annealing activity with eIF-4B. With Tif3p we could not find conditions under which this protein promotes RNA duplex melting. There might be other proteins involved and required for RNA duplex melting in yeast.

At which stage in translation initiation are RNA annealing and RNA strand exchange activities required? It has been known for a long time that eIF-4B is required for mRNA binding to ribosomes. More recently, eIF-4B was shown to stimulate eIF-4A-mediated RNA secondary structure melting *in vitro*. RNA secondary structure melting in translation initiation is assumed to happen prior to binding of 40S pre-initiation complexes to mRNA or during the scanning process. An involvement of eIF-4B in RNA secondary structure melting is reinforced by the observation that its yeast homologue, Tif3p, preferentially stimulates *in vivo* the translation of mRNA with secondary structure in the 5′-UTR. Besides melting secondary structures, RNA annealing and RNA strand exchange could also play a role in scanning and, perhaps, in the AUG recognition process. During the scanning process, the anticodon of the Met-tRNA$_i$ moiety of the ternary complex (eIF-2·GTP·Met-tRNA$_i$) must be checked constantly for base pairing with the mRNA. This is inferred from the finding that base pairing between the AUG initiation codon and the anticodon of the initiator tRNA is a major determinant for AUG codon recognition (Cigan *et al.*, 1988). Sequences flanking the initiator AUG codon contribute significantly to recognition (Kozak, 1986; Boeck and Kolakofsky, 1994; Grünert and Jackson, 1994). For steric reasons, it is unlikely that Met-tRNA$_i$ recognizes the flanking sequences. However, rRNA sequences might base-pair with mRNA sequences (Lagúnez-Otero, 1993). This would require attempts by the scanning 40S ribosome to anneal the anticodon of Met-tRNA$_i$ with the initiator AUG codon and rRNA sequences with mRNA sequences flanking the AUG codon. Unproductive complexes would have to be melted until a stable (mRNA·rRNA·Met-tRNA$_i$) complex is formed. To illustrate this, we present the following model (Figure 7): (i) annealing of RNAs would be mediated by eIF-4B homodimers binding to rRNA and mRNA; (ii) unproductive complexes would be melted by association of eIF-4A/ATP with eIF-4B on the ribosome and (iii) ATP hydrolysis would lead to the dissociation of eIF-4A followed by eIF-4B-catalysed reannealing of RNAs until a stable complex involving AUG codon–anticodon base pairing is formed. A prominent feature of this model is base pairing between rRNA and mRNA during the scanning process. This should be tested experimentally.

Materials and methods

Manipulation of yeast cells

Yeast cells were transformed using the lithium acetate method (Ito *et al.*, 1983).

DNA manipulations

Unless indicated otherwise, DNA manipulations were carried out according to Sambrook *et al.* (1989).

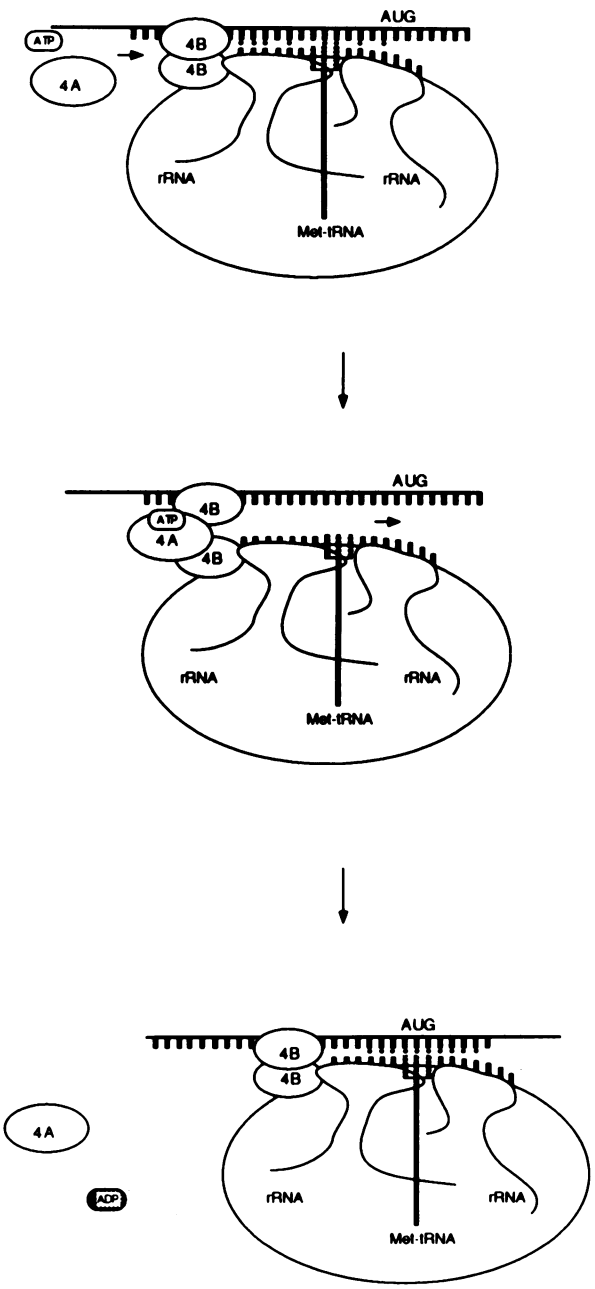

Fig. 7. Model of eIF-4B and eIF-4A involvement in scanning and AUG recognition. Initial mRNA recognition and binding to the 40S pre-initiation complex, which also requires eIF-4A and eIF-4B in addition to eIF-4F, is not depicted in this model.

Construction of TIF3 derivatives with 6× histidine tag

The centromeric plasmid p301-HIS3/GAL-TIF3 (Altmann *et al.*, 1993) was used as a template for PCR amplification of the *TIF3* gene with the 5′ primer MA105 (CCGGATCCTGAAAGGAAGAAAAA**TG**GCTC-CACCAAAG) and the 3′ primer C-His6 tag (GCGAATTCCTAA**TGGT-GATGGTGATGGTG**TTTCTTACCAACAAC). The resulting DNA fragment contained at its 5′ end a *Bam*HI restriction site (underlined in primer MA105), an AUG translation start codon (bold in primer MA105) followed by the entire *TIF3* ORF with six histidine codons at the end (bold in primer C-His6 tag) and an *Eco*RI restriction site (underlined in primer C-His6 tag). The amplified DNA fragment was cut with *Bam*HI and *Eco*RI and ligated into plasmid p301-HIS3/GAL-TIF3 from which the *TIF3* ORF had been eliminated by *Bam*HI–*Eco*RI double digestion and purification by agarose gel electrophoresis. The resulting plasmid p301-HIS3/GAL-TIF3-His6 encodes Tif3p under the control of the GAL1 promoter with a C-terminal histidine tag. It was introduced into the yeast strain *RCB-1C* (*TIF3* null allele) (Coppolecchia *et al.*, 1993); Tif3–His6p was expressed by cultivation in YP medium (1% yeast extract, 2% peptone) containing 2% galactose.

Purification of His6×-tagged protein

About 50 g of cells were resuspended in 75 ml of buffer A containing 50 mM sodium phosphate buffer (pH 8.0), 1 M NaCl, 5 mM imidazole, 5 mM β-mercaptoethanol, protease inhibitor cocktail (to give a final concentration of 1 μg/ml antipain, aprotinin, chymostatin, pepstatin) and homogenized with 50 g glass beads in a bead-beater (Biospec Products) for 3 min at 0°C. All subsequent steps were carried out at 4°C. The homogenates were centrifuged at 7500 *g* for 15 min and the supernatants applied to a 1 ml Ni^{2+} NTA column (Qiagen) equilibrated with buffer A. Bound protein was eluted stepwise with 10 ml of buffer containing 20 mM Tris–HCl, pH 7.5, 100 mM KCl, 5 mM β-mercaptoethanol, protease inhibitor cocktail and increasing imidazole concentrations of 20, 50 and 250 mM. Most of the Tif3–His6p bound to the column was eluted between 50 and 250 mM imidazole. For further purification, the imidazole eluate (diluted to 50 mM KCl) was bound to a 1 ml DEAE column (equilibrated with 20 mM Tris–HCl, pH 8.0, 50 mM KCl, 0.1 mM EDTA, 5 mM β-mercaptoethanol, protease inhibitor cocktail). Tif3–His6p eluted from the DEAE column at 150 mM KCl and was separated efficiently from contaminating proteins which co-eluted from the Ni^{2+} column. The concentration of Tif3–His6p was determined photometrically at 280 nm using a calculated molar extinction coefficient for Tif3p of 68 990.

Polyclonal antibodies against Tif3p were obtained by immunizing female rats with 150 μg Tif3–His6p in 200 μl of 50% complete Freund's adjuvant. The injection was repeated twice with incomplete Freund's adjuvant at intervals of 10 days. The rats were killed by cervical dislocation, blood collected from the heart, centrifuged and serum stored in aliquots at –70°C. SDS–polyacrylamide gels were run according to Anderson *et al.* (1973) and Western blots were carried out according to Towbin *et al.* (1979).

Purification of recombinant eIF-4B and eIF-4A

Recombinant eIF-4B and eIF-4A were expressed in *Escherichia coli* and purified according to Pause and Sonenberg (1992).

In vitro translation

Extracts from the yeast strain *RCB-1C* were prepared and cell-free translation performed as described earlier (Altmann *et al.*, 1985).

Determination of ribosome and Tif3p concentration

Ribosome concentrations were measured from the A_{260} absorbance of 40, 60 and 80S peaks after sucrose gradient fractionation of yeast cell extracts. Tif3p concentrations were determined by measuring absorbance at 280 nm (molar extinction coefficient = 68 990). Known amounts of pure Tif3–His6p were run as standards on Western blots to determine Tif3p concentrations in extracts.

Sucrose gradient analysis

The plasmid pJII2 (Sleat *et al.*, 1987; Altmann *et al.*, 1990) was cut with the restriction enzyme *Pvu*II and *in vitro* transcribed in the presence of [α-^{32}P]GTP and m^7GpppG as described (Altmann *et al.*, 1988). The specific activity of the capped RNA was ~240 000 c.p.m./pmol. Labelled RNA (1.25 pmol) was incubated in 12 μl *RCB-1C* extract (0.5–0.7 A_{260} units) at 20°C under translation conditions (Altmann *et al.*, 1985) with 700 μM unlabelled methionine for 3 min. Reactions were stopped by the addition of 38 μl of cold buffer A (20 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$) and loaded onto 4 ml 5–30% sucrose gradients in buffer A. Gradients were centrifuged at 370 000 *g* for 90 min at 4°C, fractions (170 μl) collected and counted in a liquid scintillation counter.

RNA binding, annealing and helicase activity

Reactions were carried out with the synthetic RNA substrates described before (Méthot *et al.*, 1994): a 41 nucleotide RNA strand (strand 1; labelled with [α-^{32}P]GTP) and a 68 nucleotide RNA (strand 2) capable of forming a 14 bp heteroduplex (duplex 1/2).

For RNA binding, 450 fmol of ^{32}P-labelled strand 1 (45 c.p.m./fmol) was incubated with different amounts of Tif3–His6p in 20 mM Tris–HCl (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin and 0.1 U/μl RNasin for 5 min at 25°C. The incubation mixtures (10 μl final volume) were then passed through nitrocellulose filters (Millipore HA, 0.45 μm pore size, pre-wetted with incubation buffer) under vacuum, the filters washed with incubation buffer, dried and counted in a liquid scintillation counter.

For RNA annealing, strand 1 and 2 were allowed to hybridize in 10 mM Tris, pH 7.5, 0.1 mM EDTA, 50 mM NaCl (5 min incubation at 95°C and slow cooling down to 40°C), duplex RNA 1/2 was purified from non-denaturating 8% polyacrylamide gels (run in 0.5× Tris–borate buffer at a constant 200 V for 1.5 h), extracted by incubating for 1–2 h with a 10-fold volume (compared with the gel volume) of 0.5 M ammonium acetate, 1 mM EDTA and 0.1% SDS, and ethanol precipitated. Annealing was performed with 75 fmol of RNA substrate (45 c.p.m./fmol) in a final volume of 10 µl in a buffer containing 20 mM HEPES, pH 7.2, 50 mM KCl and 0.2 U/µl RNasin, at 30°C for 20 min (unless stated otherwise).

RNA helicase assays were performed with 40 fmol of labelled RNA duplex in a final volume of 20 µl of unwinding buffer [20 mM HEPES–KOH, pH 7.2, 2 mM DTT, 5% glycerol, 0.5 mM $Mg(OAc)_2$, 0.5 mM ATP, 75 mM KCl, 0.20 U/µl RNasin] for 20 min at 37°C.

Reactions were stopped by adding 10 µg proteinase K, 0.1% SDS and incubating at 30°C for 5 min. Reaction products were separated on 15% SDS–polyacrylamide gels (Anderson *et al.*, 1973) run at 25 mA for 1.5 h. Gels were dried and exposed to X-ray films.

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