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Translation in *Saccharomyces cerevisiae*: Initiation factor 4A-dependent cell-free system

(polyclonal antibody/protein purification)

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ABSTRACT **Yeast *Saccharomyces cerevisiae* genes *TIF1* and *TIF2* (translation initiation factor) encode a protein tentatively called translation initiation factor (Tif) due to the similarity of its amino acid sequence and its molecular weight to mammalian eukaryotic initiation factor 4A. To clarify whether Tif is involved in translation, we produced an affinity-purified anti-Tif antibody by using Tif isolated from a Tif-overproducing yeast strain as immunogen and an *Escherichia coli* strain expressing Tif from an expression vector to provide the extract for affinity purification of the antibody. By using chromatographic procedures and the affinity-purified anti-Tif antibody as probe to identify Tif-containing fractions, we purified Tif from wild-type yeast cells. When yeast cells containing the only *TIF1* gene on a plasmid under the control of the galactose-inducible *CYC1–GAL10* promoter were grown in medium containing glucose as the carbon source, the production of Tif was shut off and growth was arrested. Lysates made from these cells were inactive in *in vitro* translation. Addition of Tif to these lysates restored *in vitro* protein synthesis. These results show that Tif is a translation factor, the yeast homologue of mammalian translation initiation factor 4A.**

According to current models of translation initation in eukaryotes, initiation factors recognize and bind to the 5′ terminal cap structure of mRNA and melt RNA secondary structure. Ribosomes then bind to or near the 5′ end of the mRNA and reach the initiator AUG codon by scanning the mRNA in the 5′ to 3′ direction (for reviews, see refs. 1–3). A central role in these reactions is played by the mammalian eukaryotic initiation factor 4A (eIF-4A, ref. 4), an initiation factor that binds ATP (5) and mRNA (6) and is believed to act as an RNA helicase (7). The cloning and sequencing of mouse eIF-4A cDNA (8) led to the discovery of a second form of eIF-4A in mouse cells (9) and a number of eIF-4A-related proteins in other species (10–14). Two genes encoding a protein strongly related to mouse eIF-4A were isolated from the yeast *Saccharomyces cerevisiae* and tentatively termed *TIF1* and *TIF2* (translation initiation factor genes 1 and 2, respectively; refs. 15 and 16). Since some of the eIF-4A-related proteins are probably not translation initiation factors (Tifs) (10, 11, 13), it is important to clarify whether Tif is a translation factor. Here, we show that Tif is a translation factor, the yeast homologue of mouse eIF-4A, and describe the construction of a crude cell-free eIF-4A-dependent system suitable for the study of eIF-4A function in yeast. The development of assay systems for eIF-4A activity in *S. cerevisiae* should lead to an understanding of eIF-4A function and contribute to the elucidation of RNA helicase function in general.

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EXPERIMENTAL PROCEDURES

Construction of Yeast Strain PL49. Strain PL49 (**a**, *tif1::HIS3, tif2::URA3, ade2-1, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, can 1-100*) was obtained from meiotic analysis of the diploid *PLD1* (*mat*α/*mat***a**, *TIF1/tif1::HIS3, tif2::URA3/TIF2, ade2/ade2, his3/his3, leu2/leu2, can1-100/can1-100*) carrying the *TIF1* gene on plasmid pFL39 (kindly provided by F. Lacroute, Gif-sur-Yvette, France) under the *CYC1–GAL10* promoter (16). Plasmid pFL39 is derived from plasmid pUC19 (17). It carries at coordinate 629 a *Bgl* II fragment coding for *TRP1* and at coordinate 747 a *Cla* I fragment coding for an *ARS* sequence and the centromere of *S. cerevisiae* chromosome VI. The *Hin*dIII site in *TRP1* was destroyed by site-directed mutagenesis. A *Hin*dIII–*Bam*HI fragment coding for *URA3* and the *CYC1–GAL10* promoter (18) was inserted into the polylinker. In the resulting plasmid, *Bam*HI, *Sma* I, *Kpn* I, *Sac* I, and *Eco*RI sites are available for cloning a gene under the control of the *CYC1–GAL10* promoter. In this plasmid, digested with *Sma* I and *Sac* I, we inserted a *Hae* III–*Sac* I fragment containing the open reading frame and 3′ untranslated sequences of the *TIF1* gene (15). The resulting plasmid was termed pGAL-TIF1.

Preparation of Anti-Tif Antibody. *Preparation of Tif. S. cerevisiae* strain PL49 containing a plasmid carrying the *TIF1* gene under the control of the galactose-inducible *CYC1–GAL10* promoter was grown in 1% yeast extract/2% (wt/vol) peptone/2% (wt/vol) galactose (YPGal) overnight (A_{600} = 1). Under these conditions, strain PL49 overproduced Tif. Cells (80 mg) were collected by centrifugation at 2000 × *g* for 5 min, lysed with NaOH (19), taken up in SDS/PAGE sample buffer, and fractionated by SDS/PAGE. Tif-containing regions of the gels were determined by Coomassie brilliant blue staining of small sections of the gels and comparison with the banding pattern obtained with wild-type yeast. Tif (and other proteins of the same size) was electroeluted from gels using an Electro-Eluter model 422 (Bio-Rad) according to the manufacturer's instructions.

Immunization. Approximately 35 μg of protein in 250 μl of elution buffer was mixed with an equal volume of Freund's complete adjuvant and injected intraperitoneally into two mice (BALB/cJ, female). The injections were repeated after 10 days with Freund's incomplete adjuvant. Then 15 days later, blood was collected from the heart of a freshly killed animal, allowed to clot, and clarified by centrifugation. The serum was stored at −20°C.

Affinity purification of antibody. Mouse serum, diluted 1:200 with 0.5% bovine serum albumin in 20 mM Tris·HCl, pH 7/100 mM NaCl (TBS) was reacted with the Tif-containing region of Western blots containing total *Esche-*

Abbreviations: eIF-4A, eukaryotic translation initiation factor 4A; Tif, translation initiation factor; CAT, chloramphenicol acetyltransferase.

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richia coli protein from strain HB101 (about 2 mg of protein) expressing Tif from plasmid pET-3b (20). Antibody bound to Tif on the nitrocellulose was eluted twice with 1 ml of 0.1 M glycine hydrochloride (pH 2.8), neutralized with 1 M NaOH, and diluted in 5 ml of 0.5% bovine serum albumin/TBS.

For the expression of Tif in *E. coli*, the *Hae* III–*Bam*HI fragment of the *TIF1* gene [positions 574–2358, containing the entire open reading frame and 3′ noncoding sequences (15)] was ligated to a 12-mer *Bam*HI linker and inserted into the *Bam*HI site of pET-3b (20). Production of Tif protein from the T7 promoter of pET-3b was induced by infection of cells with the phage CE6 for 2.5 hr (bacteria/phage ratio = 1:1, ref. 20). From this plasmid, Tif was expressed as a hybrid protein with 22 additional amino acids at its amino terminus.

SDS/PAGE and Western Blotting. SDS/PAGE (21) and Western blotting (22) were performed as described. Western blots were reacted with affinity-purified antibody in TBS containing 0.5% bovine serum albumin for 2–4 hr or overnight, decorated with rabbit anti-mouse antibody coupled to horseradish peroxidase (Dako, Copenhagen) for 1 hr, and stained with TBS containing 0.02% chloronaphthol and 0.01% H_2O_2.

Purification of Tif. *Preparation of post-ribosomal supernatant. S. cerevisiae* strain ABYS (**a**, *pra1, prb1, prc1, prs1, ade*) (23) was grown in 1% yeast extract/2% peptone/2% glucose (YPD) overnight at 30°C. Cells were pelleted for 5 min at 2000 × *g* and washed in distilled H_2O. Fifty grams of cells (11 liters, A_{600} = 3.5) were suspended in 200 ml of 20 mM Hepes, pH 7.4/100 mM KCl/5 mM $MgAc_2$/1 mM phenylmethylsulfonyl fluoride/14 mM 2-mercaptoethanol and homogenized with 1 vol of glass beads (0.4 mm in diameter) in a CO_2-cooled Braun homogenizer. The homogenate was separated from glass beads by filtration and centrifuged at 8000 × *g* for 10 min at 4°C. The supernatant was centrifuged in a TST41.14 rotor (Centrikon) at 40,000 rpm for 5 hr at 4°C (K-factor = 132). The supernatant (post-ribosomal supernatant) was frozen at −70°C.

Chromatographic procedures. All chromatography steps were performed at 8°C. Post-ribosomal supernatant (1 g of total protein) was applied to a Whatman DE-52 column (2.5 × 23 cm), equilibrated with 20 mM Tris·HCl, pH 7.4/100 mM KCl/0.1 mM EDTA; the column was washed with the same buffer and material was step-eluted with Tris buffer containing 150 and 250 mM KCl. Tif was eluted at 250 mM KCl. Tif was precipitated with ammonium sulfate (75% of saturation), centrifuged at 17,000 × *g* for 15 min at 4°C, dissolved in 5 ml of 20 mM Tris·HCl, pH 7.6/0.1 mM EDTA/50 mM KCl, and dialyzed against this buffer (400 vol for 2 hr).

The protein mixture was applied to a Fractogel TSK 650-DEAE (1 × 15 cm) column equilibrated with 20 mM Tris·HCl, pH 7.6/50 mM KCl. The column was washed with 20 mM Tris·HCl, pH 7.6/130 mM KCl and a linear salt gradient (130–300 mM KCl) was applied in 25 min at 2 ml/min. Tif was eluted at 150–170 mM KCl. Gradient fractions containing Tif were pooled, diluted to 50–100 mM KCl with 20 mM Tris·HCl (pH 7.6), and applied to a Pharmacia Mono-Q (5/5) column equilibrated with 20 mM Tris·HCl (pH 7.6). The column was washed with 150 mM KCl and Tif was eluted with 200 mM KCl at 1.5 ml/min, 25–35 min after equilibration with the last salt concentration. Fractions containing Tif were concentrated by ammonium sulfate precipitation (75% of saturation). The protein precipitate was dissolved in 0.5 ml of 20 mM Hepes, pH 7.2/100 mM KCl and dialyzed against the same buffer. Subsequently, 200 μl was chromatographed on a Pharmacia Superose 12 (10/30) column equilibrated in 20 mM Hepes, pH 7.2/100 mM KCl.

Assay for Tif. Aliquots of column fractions were applied to a nitrocellulose sheet (dot blotting) or electrophoresed on SDS gels and electrophoretically blotted to nitrocellulose sheets (Western blot). Nitrocellulose-bound protein was then reacted with affinity-purified antibody (see above).

Preparation of Tif-Dependent Cell-Free Extract and Cell-Free Translation. *Preparation of extracts and cell-free translation.* Strain PL49 was grown in YPGal overnight at 30°C ($A_{600} \approx 1.0$), centrifuged at 1700 × *g* for 5 min. Cells were suspended in YPD and grown at 30°C for 7–8 hr (about four generations). Growth was monitored by measuring A_{600}. When growth slowed ($A_{600} \approx 2.0$), cells were harvested. Cell-free extracts were prepared and cell-free translation was performed as described (24, 25).

Preparation of mRNA. Total yeast RNA from strain GRF18 was prepared as described (25). Chloramphenicol acetyltransferase (CAT) mRNA was transcribed *in vitro* from the plasmid pSP64 (containing the CAT open reading frame and kindly provided by N. Sonenberg, McGill University, Montreal) with SP6 polymerase and capped with vaccinia guanylyltransferase as described (26).

RESULTS

Experimental Strategy. Two genes encoding identical proteins were isolated from the yeast *S. cerevisiae* and the encoded protein was found to be similar to eIF-4A (15, 16). Since a whole family of eIF-4A-related proteins exist in several species (10–14), it was important to determine whether this protein (tentatively called Tif) is a translation factor. To answer this question, we set out to produce an anti-Tif antibody, to purify Tif from yeast cells using the antibody as a probe, and to construct yeast cells in which Tif production could be shut off. Lysates from such cells could be checked for a possible defect in protein synthesis.

Conditional System for Tif Expression. Tetrad analysis of the diploid strain PLD1 having the *TIF1* and *TIF2* genes disrupted showed that spores containing both genes disrupted could only be obtained if a plasmid carrying the *TIF1* gene was present in the diploid cell prior to sporulation (16). Using strain PL49, derived from such a spore, we made a conditional system for Tif expression by having the *TIF* gene under the control of the galactose-inducible *CYC1–GAL10* promoter on the centromeric plasmid pFL39. Since the Tif function encoded by this plasmid was the only source for Tif in strain PL49, this strain could grow only on galactose-containing medium, where it actually overproduced Tif (results not shown). If, however, this strain was transferred into glucose-containing medium, growth slowed strongly after four to six generations. Thus, cells that became depleted of Tif activity could no longer grow.

Generation of an Anti-Tif Antibody. By comparison of SDS/PAGE patterns from lysates of wild-type yeast cells with those from the strain PL49, which overproduced Tif on galactose-containing medium, Tif-containing regions could be localized on gels and excised, and Tif could be eluted. Mice were immunized with gel-eluted Tif. Anti-Tif antibodies from the sera of these mice were affinity-purified on Western blots of extracts of *E. coli* cells expressing Tif from a plasmid regulated by the phage T7 promoter. Affinity-purified anti-Tif antibody reacted with a protein of the expected molecular mass for Tif (42 kDa) in yeast cells (Fig. 1, lane 1) and in *E. coli* cells harboring the *TIF* gene inserted in the correct orientation behind the T7 promoter (Fig. 1, lane 2). It did not react, however, with *E. coli* extracts derived from cells with the *TIF* gene inserted in the wrong orientation behind the T7 promoter that could thus not synthesize Tif protein (Fig. 1, lane 3). This shows that our affinity-purified antibody reacts specifically with Tif.

Purification of Tif Protein. Tif was purified from post-ribosomal supernatants of wild-type yeast by DEAE-cellulose chromatography, by medium-pressure liquid chromatography on anion exchangers, and by size-separation.

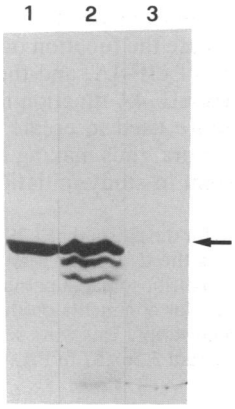

FIG. 1. Characterization of affinity-purified anti-Tif antibody. Proteins were fractionated by SDS/PAGE, transferred electrophoretically to nitrocellulose, and reacted with anti-Tif antibody. Lanes: 1, ≈85 μg of total protein from strain PL49 grown overnight in YPGal to an A_{600} of 1; 2, 85 μg of total *E. coli* protein from strain HB101 containing the *TIF* gene inserted in the correct orientation for expression in the plasmid pET-3b; 3, 85 μg of total *E. coli* protein from strain HB101 containing the *TIF* gene inserted in the wrong orientation for expression in pET-3b. The arrow indicates the position of ovalbumin (43 kDa).

During purification, fractions containing Tif were identified by dot blotting or Western blotting and reaction with the affinity-purified antibody. Tif was ≈750-fold enriched and ≈80% pure, as judged by SDS/PAGE analysis (Fig. 2, lane 1). From these data, we calculate that Tif amounts to ≈0.1% of total yeast protein or 2–3 mol of Tif per mol of ribosomes. Tif always formed a double band, but the relative abundance of the two bands varied from preparation to preparation. Both bands are Tif as shown by their reaction with the affinity-purified anti-Tif antibody (Fig. 2, lane 2). At present, we don't know what sort of modification of Tif is involved in double-band formation.

Tif-Dependent Cell-Free Translation System. To resolve the question of whether Tif is involved in translation, we used strain PL49, in which the only copy of the *TIF* gene is on a plasmid under the control of the galactose-inducible *CYC1–GAL10* promoter. In glucose-containing medium, these cells stop producing Tif and growth is slowed after 6–8 hr (three to four generations). Lysates prepared from cells grown overnight in galactose-containing medium and transferred to glucose-containing medium for one generation were fully active in *in vitro* translation (results not shown). Lysates

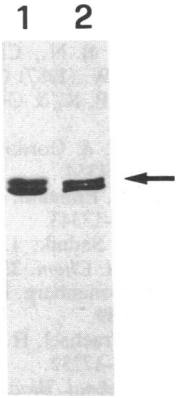

FIG. 2. Characterization of purified Tif. Purified Tif (Superose 12 fraction) was fractionated by SDS/PAGE and stained with Coomassie brilliant blue or transferred electrophoretically to nitrocellulose and reacted with anti-Tif antibody. Lanes: 1, 5 μg (Coomassie blue stained); 2, 0.5 μg (Western blot). The arrow indicates the position of ovalbumin (43 kDa).

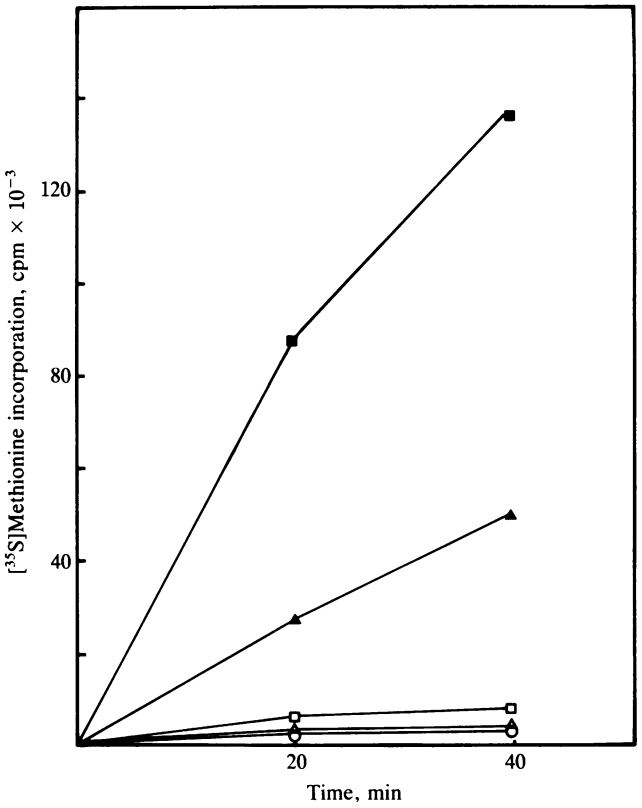

FIG. 3. Cell-free translation. Extract of strain PL49 was digested with micrococcal nuclease (25), reaction mixtures (20 μl) were incubated at 25°C, and 5-μl aliquots were analyzed for [35S]methionine incorporation (25). ○, No addition; △, plus 10 μg of total yeast RNA; □, plus 50 ng of CAT mRNA; ▲, plus 10 μg of total yeast RNA plus 1 μg of purified Tif (Mono Q fraction); ■, plus 50 ng of CAT mRNA plus 1 μg of purified Tif (Mono Q fraction). Aliquots (5 μl) were analyzed for [35S]methionine incorporation (25).

derived from cells in the slow growth phase, however, were inactive when supplied with either total yeast RNA or *in vitro* transcribed and capped CAT mRNA (Fig. 3 and Fig. 4, lanes 1 and 3). Translation could be restored in these lysates by addition of purified Tif (Fig. 3 and Fig. 4, lanes 2 and 4). In the experiments shown here, Tif purified to the Mono Q step was used. Control experiments showed that Tif purified to

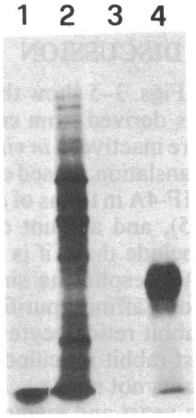

FIG. 4. Analysis of translation products. Aliquots (10 μl) of translation mixtures were incubated for 40 min (Fig. 3) and were analyzed by SDS/PAGE and fluorography (27). Lanes: 1, 10 μl of translation mixture containing 10 μg of total yeast RNA; 2, as in lane 1 plus 1 μg of purified Tif (Mono Q fraction); 3, 10 μl of translation mixture containing 50 ng of CAT mRNA; 4, as in lane 3 plus 1 μg of purified Tif (Mono Q fraction).

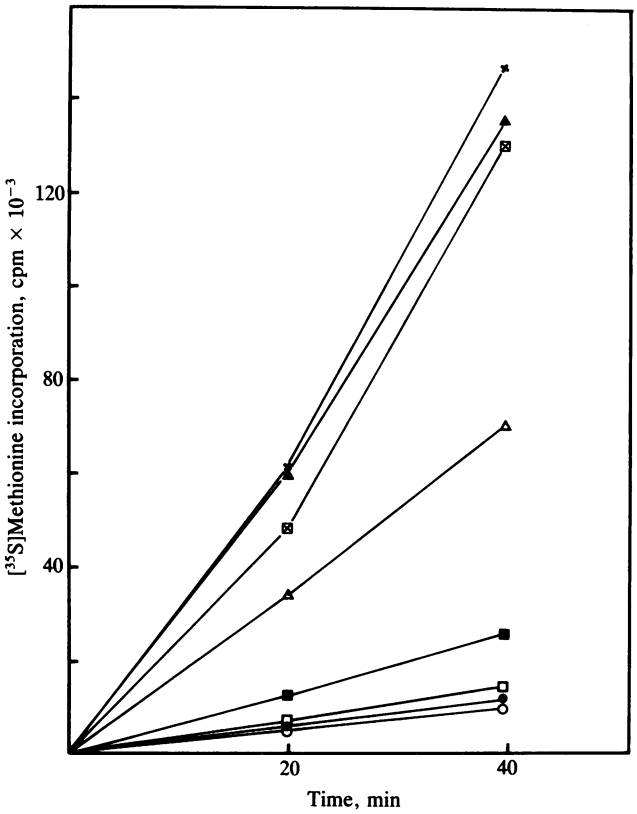

FIG. 5. Cell-free translation: Titration of Tif. Extract of strain PL49 was digested with micrococcal nuclease, reaction mixtures (20 μl) were incubated at 25°C, and 5-μl aliquots were analyzed for [35S]methionine incorporation (25). ○, No addition; ●, plus 10 μg of total yeast RNA. The other incubation mixtures contained in addition to 10 μg of total yeast RNA the following components. □, 50 ng of Tif; ■, 250 ng of Tif; △, 500 ng of Tif; ▲, 1 μg of Tif; ×, 1.5 μg of Tif; ⊠, 2 μg of Tif. Tif protein was purified to the Mono Q step.

the Superose 12 step behaved identically. About 1.5 μg of Tif or 4 pmol of Tif per pmol of ribosomes gave maximal translational activity with total yeast RNA as template (Fig. 5). The stimulation of translation was 15- to 20-fold. Translation extracts prepared from wild-type yeast cells were also stimulated 3-fold by addition of purified Tif (result not shown). These results show that Tif is essential and limiting for translation in our yeast extracts.

DISCUSSION

The data presented in Figs. 3–5 show that Tif is a translation factor, since (*i*) lysates derived from cells that switched off the production of Tif are inactive in *in vitro* translation and (*ii*) purified Tif restores translation. Based on the similarity of Tif with the mammalian eIF-4A in terms of amino acid sequence, molecular mass (9, 15), and amount of factor present per ribosome (28), we conclude that Tif is the yeast homologue of mammalian eIF-4A. Despite the similarity of yeast and mammalian eIF-4A, our affinity-purified anti-Tif antibody does not react with rabbit reticulocyte eIF-4A and a monoclonal antibody against rabbit reticulocyte eIF-4A (30) does not react with Tif (results not shown). A similar observation was made earlier with yeast and mammalian eIF-4E (25).

eIF-4A is believed to be involved with other initiation factors in ATP-dependent unwinding of secondary structure in the 5′ untranslated region of mRNA (1, 2, 7), allowing the ribosome to scan mRNA for the initiator AUG codon (3, 29). The eIF-4A-dependent translation system described above, to our knowledge, is the first initiation factor-dependent crude translation system from yeast. This system should be of great value to elucidate the function of eIF-4A, structure–function relationships of eIF-4A, and the mRNA structural determinants requiring eIF-4A function for translation. Furthermore, the procedure used to create this lysate may be applicable to other factors, thus making the yeast system an attractive model system to study initiation factor function.

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