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Translation Initiation Factor 4A from *Saccharomyces cerevisiae*: Analysis of Residues Conserved in the D-E-A-D Family of RNA Helicases

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The eukaryotic translation initiation factor 4A (eIF-4A) possesses an in vitro helicase activity that allows the unwinding of double-stranded RNA. This activity is dependent on ATP hydrolysis and the presence of another translation initiation factor, eIF-4B. These two initiation factors are thought to unwind mRNA secondary structures in preparation for ribosome binding and initiation of translation. To further characterize the function of eIF-4A in cellular translation and its interaction with other elements of the translation machinery, we have isolated mutations in the *TIF1* and *TIF2* genes encoding eIF-4A in *Saccharomyces cerevisiae*. We show that three highly conserved domains of the D-E-A-D protein family, encoding eIF-4A and other RNA helicases, are essential for protein function. Only in rare cases could we make a conservative substitution without affecting cell growth. The mutants show a clear correlation between their growth and in vivo translation rates. One mutation that results in a temperature-sensitive phenotype reveals an immediate decrease in translation activity following a shift to the nonpermissive temperature. These in vivo results confirm previous in vitro data demonstrating an absolute dependence of translation on the *TIF1* and *TIF2* gene products.

Translation initiation is assumed to be a key step in the regulation of translation (for reviews, see references 31, 40, 49, and 53). Most investigations of translation have used in vitro systems which reconstitute translation by using purified components from higher eukaryotes. The availability of an in vitro system from *Saccharomyces cerevisiae* allows the study of cloned initiation factors, using extracts prepared from cells deficient in translation factors (2, 4). Yeast genetics also offers possibilities for studying the interaction between mutant initiation factors or between mRNA and the initiation machinery by isolating suppressors. For example, suppressors of initiation codon mutants have been isolated and found to encode the α and β subunits of eukaryotic initiation factor 2 (eIF-2) (9, 13).

Two alternate models have been proposed to describe the first step in translation initiation in higher eukaryotes. In one model, the 5′ cap structure is recognized by a preformed cap-binding complex consisting of a 24-kDa cap-binding protein (eIF-4E), a 220-kDa protein, and a 46-kDa protein (eIF-4A) (19). The other model proposes that the cap is recognized by eIF-4E alone and subsequent binding of p220 and eIF-4A (15). In a next step, the cap-binding complex is joined by another factor, eIF-4B, and additional eIF-4A, which together are postulated to unwind mRNA secondary structures in the presence of ATP (41). The mRNA adopts a relaxed conformation allowing the 43S preinitiation complex, composed of the 40S ribosomal subunit, the initiator methionyl-tRNA, eIF-2, and GTP, to bind and scan for the initiator AUG. Binding of this preinitiation complex to mRNA is thought to be assisted by eIF-4B (28). The initiator methionyl-tRNA bearing the 3′-UAC-5′ anticodon (8) together with eIF-2 (9, 13) is required for recognition of the initiator AUG. In a last step, the 60S ribosomal subunit binds to this complex and the initiation factors are released.

In contrast to the cap-binding proteins from higher eukaryotes, these proteins are biochemically not well defined in yeast cells. Nevertheless, it is clear that yeast cells have an eIF-4E protein (which is isofunctional to the mouse eIF-4E in vivo [1]) and a high-molecular-weight component similar to the mammalian 220-kDa protein (18). Yeast cells also have an eIF-4A protein (referred to as eIF-4A$_Y$), although this factor is not found tightly associated with the cap-binding complex (18).

eIF-4A$_Y$ is encoded by two duplicated genes, the translation initiation factor genes *TIF1* and *TIF2* (33). This feature of duplicated genes is typical for genes encoding proteins involved in translation. It has previously been found for the genes encoding eIF-5A (previously named eIF-4D [45]), the genes encoding elongation factor 1α (EF-1α) (44), and many ribosomal protein genes (38). The genes *TIF1* and *TIF2* encode exactly the same protein, and although the flanking sequences are different, disruption of either copy results in no phenotype under normal laboratory growth conditions. Disruption of both genes is lethal to the cell. In a yeast in vitro translation system, depletion of eIF-4A$_Y$ results in peptide synthesis arrest. This can be alleviated by the addition of purified eIF-4A (4), suggesting that the *TIF1* and *TIF2* gene products are directly involved in mRNA translation.

A number of proteins that are similar to eIF-4A have been described and grouped into the D-E-A-D protein family of putative RNA helicases (30). Other proteins belonging to this family have been described since (5–7, 10, 11, 26, 43, 51). Genetic and biochemical results show that some of them are involved in RNA translation or splicing. This protein family is characterized by three main domains of high homology. Two of them most likely mediate ATP binding and ATPase activity (42): the ATPase A motif, with the sequence AxxGxGKT, and the LDEAD sequence, which is a special form of the ATPase B motif characteristic of this family. This latter motif appears as DEAH in some RNA-splicing proteins. No function has yet been attributed to the third

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TABLE 1. Strains used

Strain	Genotype
S. cerevisiae	
PL49	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3 can1-100 tif1::URA3 tif2::HIS3* (33)
ASZ1	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3 can1-100*
SS1-1B	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3 can1-100* Δtif1::*HIS3* Δ*tif2*::*URA3*-Pgal-*TIF2*
SS2	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3 can1-100* Δ*tif1*::*HIS3*
SS3	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3 can1-100* Δ*tif2*::*URA3*-Pgal-*TIF2*
SS4	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3 can1-100* Δ*tif2*::*URA3*
SS5-6A	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3 can1-100* Δ*tif1*::*HIS3* Δ*tif2*::*URA3*
SS5-5C	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3 can1-100* Δ*tif1*::*HIS3* Δ*tif2*::*URA3*
SS8	*MATa/MATα ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3/ura3 can1-100/can1-100* Δ*tif1*::*HIS3*/Δ*tif1*::*HIS3* Δ*tif2*::*URA3*/Δ*tif2*::*URA3*
SS8-3A	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3 can1-100* Δ*tif1*::*HIS3* Δ*tif2*::*URA3* (pSSC120)
SS8-3B	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3 can1-100* Δ*tif1*::*HIS3* Δ*tif2*::*URA3* (pSSC120)
SS8-3D	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3 can1-100* Δ*tif1*::*HIS3* Δ*tif2*::*URA3* (pFL39-*TIF1*)
E. coli	
MC1061	*araD139* Δ(*ara-leu*)*7697* Δ*lacX74 galU galK hsr rpsL*
JM101	*supE thi* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q} ZΔM15)
TG1	Δ(*lac-pro*) *supE thi hsdD5* (F′ *traD36 proAB lacI*^{q} ZΔM15)

motif with the sequence HRIGR, but it has been suggested to be involved in the interaction with RNA (30).

Some members of this family, the mouse eIF-4A and the human p68 protein (a major nuclear antigen present in dividing hepatoma cells), have been shown to possess ATP-binding and ATPase activities as well as an RNA helicase activity (19, 22, 25, 39). In the case of mouse eIF-4A, these activities require eIF-4B (28, 41). For the Srmb protein, ATP binding and a nucleic acid-dependent ATPase activity have been shown (37). To date, no consensus sequence for RNA helicase activity has been defined. Although the D-E-A-D proteins have different functions, it is possible that they have the same or very similar mechanisms. The analysis of eIF-4A$_Y$ may help us to understand the mode of action of RNA helicases.

To understand the role of eIF-4A$_Y$ in translation initiation and to further characterize the function of the three highly homologous domains, we have undertaken site-directed mutagenesis to study the *TIF1* and *TIF2* genes. Here we report that the ATPase A and B motifs and the HRIGR domain are essential for protein function and that their sequence requirements are very strict. Chemical mutagenesis also allowed us to isolate a temperature-sensitive mutant as well as mutants that display slower growth rates. Here we describe the in vivo characteristics of these mutants.

MATERIALS AND METHODS

Media and yeast strains. Yeast media were as described by Sherman et al. (47). The strains used are described in Table 1. *S. cerevisiae* CWO4 (3) and ASZ1, an isogenic derivative of CWO4 of opposite mating type, were used for construction of the *TIF* disruptions.

For disruption of the *TIF1* gene, the *Cla*I-*Xho*I fragment of *TIF1* in strain CWO4 was replaced by the *HIS*3 gene (pUC19-Δ*tif1*::*HIS3*; Fig. 1B), resulting in SS2. Southern analysis of genomic DNA restricted with *Bam*HI with labeled pUC13-*TIF1* DNA used as a probe confirmed disruption of *TIF1*. For disruption of the *TIF2* gene, the *Pst*I fragment harboring the open reading frame was replaced by the *URA3* gene (pUC19-Δ*tif2*::*URA3*; Fig. 1C). Replacement of *TIF2* in strain ASZ1 resulted in strain SS4.

To integrate a *TIF* gene under the control of the galactose promoter into the genome, the *Hin*dIII fragment of pGAL-*TIF1* (containing the *URA3* gene and the *GAL10-CYC1* hybrid promoter [20] followed by the first third of the *TIF1* gene) was used to replace the *URA3* gene in pUC19-Δ*tif2*::*URA3* (Fig. 1C). This plasmid was then restricted with *Cla*I, which cuts in the 5′-flanking region of *TIF2*, and *Kpn*I, which cuts within the first third of the *TIF1* open reading frame, and then transformed into ASZ1 to yield SS3. This results in a hybrid gene in which the *GAL10-CYC1* promoter is followed by the 5′ part of *TIF1* and the 3′ fragment is from *TIF2*. Both *TIF2* replacements were confirmed by Southern hybridization with a pUC13-*TIF2* DNA probe.

To construct strains in which both *TIF* genes have been disrupted, we crossed strain SS2 with either SS3 or SS4. The diploid strain from the cross SS2 × SS3 was renamed SS1, and its haploid spore (with both replacements) was named SS1-1B. SS4 was transformed with the *LEU2*-containing YEp13-*TIF1* plasmid (33) and was crossed with SS2 to obtain the diploid strain SS5/YEp13-*TIF1*. After sporulation and tetrad analysis, we obtained the haploid doubly disrupted strains SS5-6A/YEp13-*TIF1* and SS5-5C/YEp13-*TIF1* (Table 1).

For screening of mutants, we constructed a diploid strain with all four *TIF* genes deleted. This strain, SS8/pGAL-*TIF1*, was obtained by mating of SS5-5C/YEp13-*TIF1* with SS5-6A/pGAL-*TIF1* and subsequent loss of YEp13-*TIF1*.

Construction of plasmids for mutagenesis. *Escherichia coli* strains are described in Table 1. Standard procedures were used in all DNA manipulations (34). For most of the constructions, the vectors pFL39 (26a) and a derivative in which both *Cla*I sites had been inactivated [pFL39(Δ*Cla*)] were used (Fig. 1A).

A hybrid *TIF1*/*TIF2* gene was constructed for the mutagenesis of the two ATPase domains. This chimera contains the 5′ region of the *TIF1* gene extending to the internal *Hin*dIII site, followed in frame by the rest of the eIF-4A reading frame and the 3′ region of *TIF2* [pFL39(Δ*Cla*)-*TIF1*/*2*; Fig. 1C]. From this plasmid, a *Bgl*II-*Pst*I fragment was excised [the *Bgl*II site is within pFL39(Δ*Cla*)] and inserted into the *Bam*HI and *Pst*I sites of the polylinker of pTZ18R (Pharmacia) for mutagenesis. After isolation of the desired mutations, the *Acc*I-*Pst*I fragment was cloned back into pFL39(Δ*Cla*)-*TIF1*/*2* to reconstruct a *TIF1* open reading frame with the desired mutations.

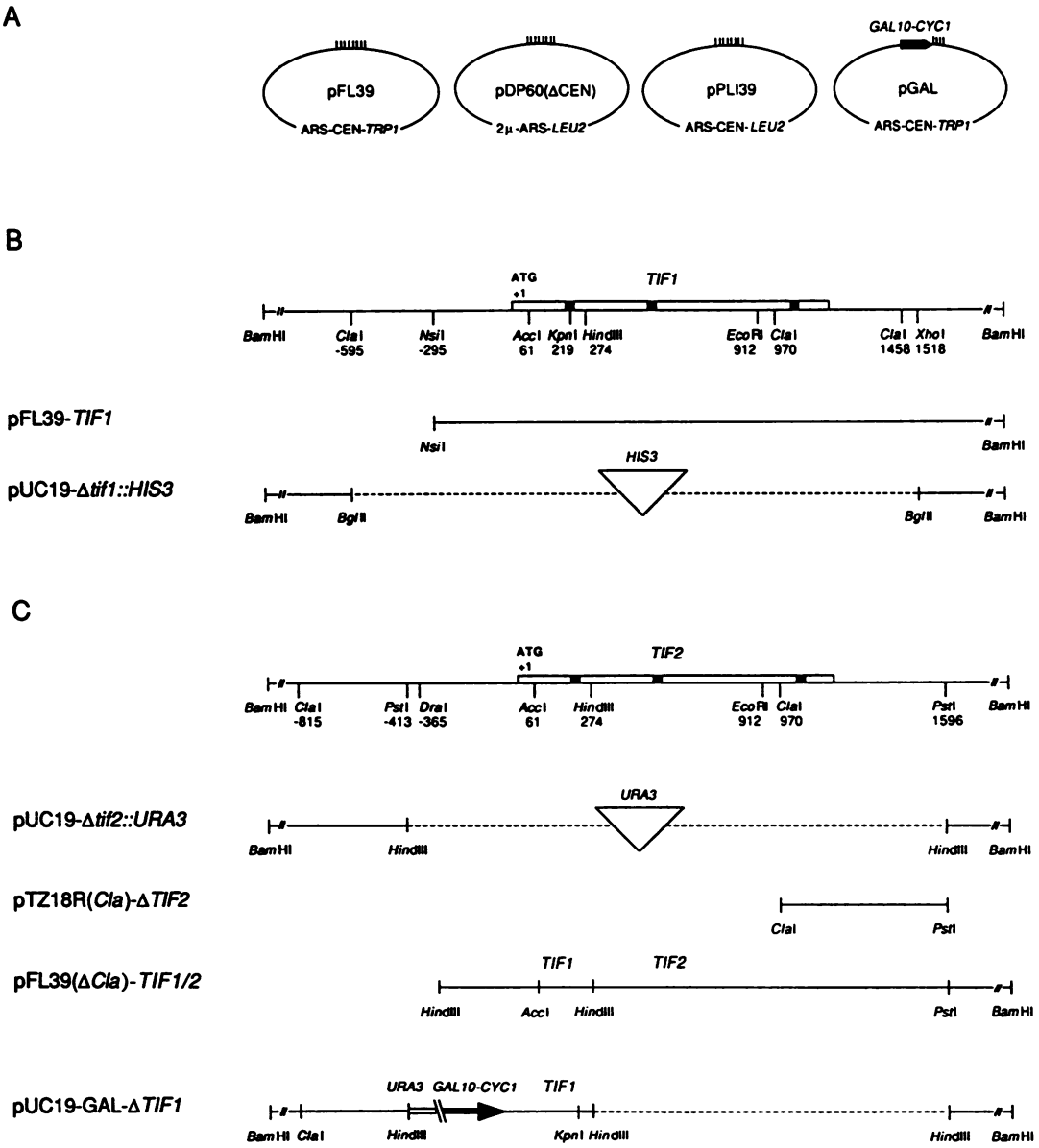

FIG. 1. (A) Schematic view of the vectors used. Plasmid pFL39 (26a) is based on pUC19 and carries the *TRP1* gene and an ARS-CEN fragment. pFL39(Δ*Cla*) is a derivative of pFL39 in which the two *Cla*I sites, flanking the ARS-CEN fragment, have been inactivated by filling in. Plasmid pDP60(ΔCEN) (38a) carries the ARS sequence from the 2μm plasmid and a *LEU2* gene. In pPLI39, the *TRP1* gene of pFL39 has been replaced by the *LEU2* gene. The solid arrow in pGAL represents the *GAL10-CYC1* promoter (4). pGAL also contains a *URA3* marker (not shown). Small bars represent the restriction sites of the polylinkers. Not shown are plasmids pTZ18R and pTZ18R(*Cla*), which are similar to pUC19 but carry also a f1 origin of replication for packaging of single-stranded DNA. In pTZ18R(*Cla*), a *Cla*I linker has been inserted into the *Bam*HI site of the polylinker. (B) Restriction map of the *TIF1* gene. Only relevant restriction sites used for plasmid constructions are shown. The three conserved domains are shown by solid boxes. They are, from left to right, the ATPase A motif (AxxGxGKT), the ATPase B motif (LDEAD), and the HRIGR motif (HRIGR). The fragments used in different plasmids are indicated below the map. The top line represents the *TIF1 Bam*HI fragment cloned into pUC13. The *Nsi*I-*Bam*HI fragment was subcloned into the *Pst*I and *Bam*HI sites of plasmids pFL39, pDP60(ΔCEN), and pPLI39 to yield pFL39-*TIF1*, pDP60(ΔCEN)-*TIF1*, and pPLI39-*TIF1*, respectively. Deletion of the *TIF1* gene and insertion of the *HIS3* gene as a *Bam*HI fragment into a *Bgl*II linker is indicated by the interrupted line with a triangle in pUC19-Δ*tif1*::*HIS3*. pGAL-*TIF1* has been described by Blum et al. (4). (C) Restriction map of the *TIF2* gene. Only relevant restriction sites used for plasmid constructions are shown. The *Acc*I sites in the 5′ upstream region of *TIF2* were omitted for simplicity. No *Kpn*I site is present in *TIF2*. The three conserved domains are shown by solid boxes. The cloned *Bam*HI fragment in pUC13 is shown on top. The different constructs for plasmids and integration in the genome are indicated below the *TIF2* map. Deletion of the *TIF2* gene and insertion of a *Hin*dIII fragment harboring the *URA3* gene are indicated by the interrupted line and the triangle in plasmid pUC19-Δ*tif2*::*URA3*. Plasmid pTZ18R(*Cla*)-Δ*TIF2* contains only the small *Cla*I-*Pst*I fragment of *TIF2* and was used for site-directed mutagenesis. In plasmid pFL39(Δ*Cla*)-*TIF1/2*, the *Hin*dIII fragment containing the 5′ third of *TIF1* was fused in frame to the 3′ portion of *TIF2*. The leftmost *Hin*dIII site originates from the polylinker from pFL39-*TIF1*. In plasmid pUC19-GAL-Δ*TIF1*, a *Hin*dIII fragment containing the *URA3* gene and the *GAL10-CYC1* promoter (represented by an open box and an arrow, respectively, not drawn to scale) followed by part of the *TIF1* open reading frame was cloned into the *Hin*dIII site of pUC19-Δ*tif2*::*URA3* instead of the *URA3* gene. The *Cla*I-*Kpn*I fragment was then used for integration.

For oligonucleotide-directed mutagenesis of the HRIGR motif, the *Cla*I-*Pst*I fragment of *TIF2* was subcloned into pTZ18R(*Cla*), a derivative of pTZ18R in which a *Cla*I linker was introduced into the *Bam*HI site [pTZ18R(*Cla*)-Δ*TIF2*; Fig. 1C]. After mutagenesis, this same fragment was replaced into pFL39(Δ*Cla*)-*TIF2*, in which the *TIF2* gene (ranging from the *Dra*I to the second *Eco*RI site, located in the polylinker) was cloned into pFL39(Δ*Cla*).

For nitrosoguanidine mutagenesis, we used pDP60 (ΔCEN)-*TIF1*, in which the *Nsi*I-*Bam*HI fragment of *TIF1* was subcloned into the *Pst*I and *Bam*HI sites of the polylinker of the vector. The vector pDP60(ΔCEN) (provided by David Pridmore) contains the 2μm autonomous replicating sequence (ARS) and the *LEU2* gene in pUC18. After isolation and sequencing of mutations, we subcloned the mutated genes into pFL39 in order to be able to compare them with the rest of the mutants.

We also constructed plasmid pPLI39, which carries the *LEU2* fragment (*Hpa*I-*Sal*I fragment from YEp13) instead of the *TRP1* gene in pFL39. For hydroxylamine mutagenesis, we used pPLI39-*TIF1* with the *Nsi*I-*Bam*HI *TIF1* fragment integrated into the *Pst*I and *Bam*HI sites of the pPLI39 polylinker.

Oligonucleotide-directed mutagenesis. Single-stranded DNA used for site-directed mutagenesis was prepared as described by Stotz and Linder (50). For mutagenesis of the ATPase A, ATPase B, and HRIGR motifs, we used the synthetic oligonucleotides 5′-ggctcaagntcaatctggtactgg-3′, 5′-catcttagangaagctgatg-3′, and 5′-gaaaactatattcacnnnatcggt agagg-3′, respectively, where n represents a mixture of all four nucleotides. For mutagenesis of aspartic acid residue 173, we used the oligonucleotide 5′-gatgaagctcatgaaatg-3′. Mutagenesis was performed by using the oligonucleotide-directed in vitro mutagenesis system from Amersham according to the manual. Mutated plasmids were transformed into *E. coli* TG1, and plasmid from individual transformants was sequenced over the entire coding sequence of the subcloned fragment. After recloning of the fragment harboring the desired mutations in the yeast plasmids, the presence of the mutation on the plasmid was again confirmed by sequencing. Finally, the plasmids were transformed into strain SS1-1B and selected on minimal medium containing galactose (SGal) without tryptophan, and several single transformants were analyzed for growth on glucose-containing medium.

Chemical mutagenesis. Hydroxylamine mutagenesis was done as described by Völker et al. (54). Plasmid pPLI39-*TIF1* was subjected to mutagenesis using hydroxylamine at a final concentration of 0.05 mg/ml. After 0, 10, 20, and 60 min, aliquots were removed from the reaction mixture and dialyzed. The DNA was precipitated with ethanol, extracted with phenol, and reprecipitated. DNA samples were then transformed into *E. coli* JM101 and directly used to isolate plasmid in small scale.

For nitrosoguanidine mutagenesis, we used pDP60 (ΔCEN)-*TIF1* in strain JM101 and followed the procedure described by Silhavy et al. (48). We observed 5% survival after 12 min of incubation at 37°C and less than 1% survival after 22 min on ampicillin plates. The plasmid DNA was isolated and transformed into PL49/pGAL-*TIF1* (4), and transformants were selected on SGal plates without leucine.

In vivo protein labeling experiments. Precultures (SS1-1B carrying the different mutant plasmids) were grown in SGal supplemented with the appropriate amino acids and bases for 2 days. To deplete the cells of the wild-type eIF-4A protein, the cultures were diluted into SD medium and grown for 20 h to an optical density at 600 nm (OD_{600}) of 0.2 to 0.5. Cells corresponding to 3 OD_{600} units were harvested by centrifugation and resuspended in 1 ml of fresh SD containing 20 μCi of [^{35}S]methionine (1,070 Ci/mmol; NEN), and incubation was continued at 30°C (or 37°C when specified) for 5 min. Cells were lysed in NaOH–β-mercaptoethanol, proteins were precipitated with trichloroacetic acid (56) and dissolved in sample buffer, and aliquots were loaded on a 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and subjected to polyacrylamide gel electrophoresis (PAGE).

RESULTS

Construction of a conditional *TIF* system. To identify mutations in the genes coding for eIF-4A, we used a conditional *TIF* system in which the chromosomal *TIF1* and *TIF2* genes of the diploid strain (SS8/pGAL-*TIF1*) had been deleted and which harbored plasmid pGAL-*TIF1* to maintain viability. This plasmid contains the *TIF1* gene under the control of a galactose-inducible, glucose-repressible promoter such that SS8/pGAL-*TIF1* cells are able to grow only on galactose medium. The use of this strain offered two main advantages: (i) deletion of all four chromosomal *TIF* genes prevents homologous recombination between chromosomal and plasmid-borne genes, and (ii) the use of a diploid strain masks the phenotypic appearance of recessive genomic mutations.

To characterize the conditional phenotype of this strain, cultures were grown to exponential phase in galactose medium and then shifted to glucose medium. Throughout the growth period, the OD_{600} was used as a measurement of cell division, and cells were plated onto galactose medium to analyze cell viability. We found that cells arrest division after approximately 30 h and that during the next 40 h, cell viability is reduced to 10%. Microscopic examination revealed that cells arrested with a diameter two to three times larger than that of the wild-type strain.

A slightly different conditional system was constructed in SS1-1B, in which a conditional copy of a chimeric *TIF1-TIF2* gene under the control of the galactose promoter was present at the genomic *TIF2* locus (together with the *URA3* marker gene). This strain behaves similarly to SS8/pGAL-*TIF1* when shifted to glucose medium and was used for the analysis of mutants isolated by site-directed mutagenesis.

Oligonucleotide-directed mutagenesis of the three conserved domains. Proteins in the D-E-A-D family contain several conserved domains. To investigate the contribution of these sequences to the function of *TIF1* and *TIF2*, we undertook a mutational analysis. A summary of all of the isolated mutations together with their in vivo phenotypes and growth rates is shown in Fig. 2.

Our investigation of the first domain, the ATPase A motif (AxxGxGKT), focused on the alanine residue at position 66 of the eIF-4A$_Y$ protein. The alanine residue at this position is found in most of the members of the family. Only Spb4 (43) and Prp5 (11) proteins have a serine at this position, whereas the splicing proteins Prp16, Prp22, and Prp2 have a glycine (5, 7, 10). This alanine residue was replaced by valine (A66V), aspartic acid (A66D) or glycine (A66G). Our results show that a valine residue at position 66 is lethal for the cell, whereas the presence of an aspartic acid results in a slower growth rate. The replacement by glycine results in almost the wild-type growth rate.

The second region of high homology is defined by the D-E-A-D motif (LDEAD), thought to represent a special

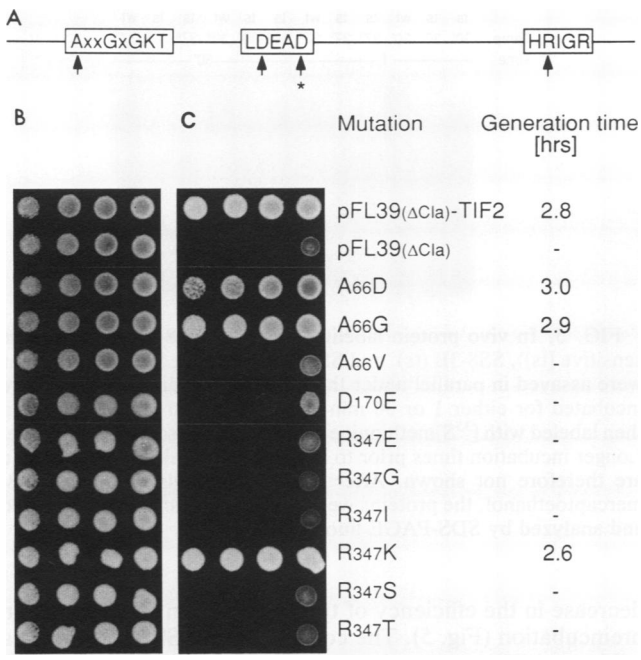

FIG. 2. Mutations in the three conserved domains and resulting phenotypes. (A) Amino acid sequences of the three motifs (not drawn to scale). The mutagenized residues are indicated by arrows. The aspartic acid residue, that is a histidine in the splicing proteins (see text), is indicated by an asterisk. (B and C) Effects of the mutations on cell growth in strain SS1-1B. Droplets of increasing dilutions of the cultures were plated on galactose-containing plates (B) and on glucose-containing plates (C). The dilution factors (from left to right) are 6,250, 1,250, 250, and 50 times. Droplets of the mutation D173H are not shown. Mutations A66D, A66G, and A66V are on pFL39(Δ*Cla*)-*TIF1/2*, whereas mutation R347K is on pFL39 (Δ*Cla*)-*TIF2*. The generation times of SS1-1B bearing pFL39(Δ*Cla*)-*TIF2*, pFL39(Δ*Cla*)-*TIF1/2*, pPLI39-*TIF1*, and pFL39-*TIF1* are 2.8, 2.7, 2.7, and 2.6 h, respectively.

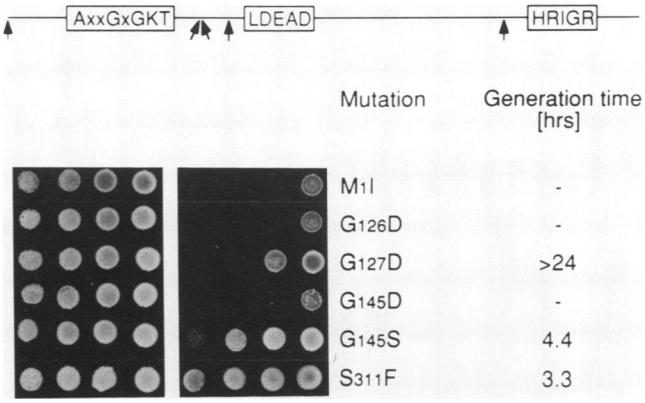

FIG. 3. Phenotypes of mutants isolated by chemical mutagenesis. Droplets, map, and generation times of the mutants are shown as in Fig. 2. Arrows indicate the mutated residues. Mutations M1I, G126D, G127D, and G145D are on pFL39-*TIF1*, whereas G145S and S311F are on pPLI39-*TIF1*.

form of the ATPase B motif. Within this sequence we mutated the first aspartic acid at position 170 to a glutamic acid (D170E). This protein is not active as determined in the conditional system in strain SS1-1B. This was an expected result since this residue is conserved not only in all of the proteins of this family but also in other ATPases (23). Because the three splicing proteins Prp16, Prp22, and Prp2 have the sequence DEAH (versus DEAD), we also decided to substitute the aspartic acid at position 173 by histidine. The resulting protein is also nonfunctional.

Within the third motif (HRIGR, suggested to be involved in the interaction with RNA), we mutagenized the first arginine residue. This amino acid is also highly conserved, as a variant with threonine instead of arginine has been found in only one protein (Prp5). Analysis of the different mutations that we constructed revealed that only the conservative substitution of the arginine residue to a lysine residue (R347K) resulted in an active protein.

To be sure that the negative phenotype of the mutant strains is not due to the selection of *trp1-1* suppressors, we also performed plasmid segregation. Upon cultivation in nonselective medium, tryptophan-negative cells appeared, confirming the presence of a plasmid in the cells.

Chemical mutagenesis of the *TIF* genes. To complement the site-directed mutagenesis approach, we also undertook random mutagenesis using either hydroxylamine or nitrosoguanidine. After mutagenesis, plasmid DNA was transformed into *E. coli* and then into the yeast strain SS8/pGAL-*TIF1* or PL49/pGAL-*TIF1*. Colonies were selected on SGal without leucine and replica plated onto glucose-containing medium at 30°C. Because of the large number of cells transferred by replica plating, the phenotype is not immediately visible. Therefore, a second replica was made in triplicate and incubated at 18, 30, and 36°C to identify temperature- or cold-sensitive mutants. Candidates that grew more slowly on glucose were picked from the parental galactose plates for further analysis. They were streaked for single colonies on SGal and tested for growth on glucose at different temperatures. DNA was isolated from the candidates that displayed an interesting phenotype, transformed into *E. coli* MC1061, which allows selection not only for ampicillin resistance but also for leucine auxotrophy, and reisolated. Finally, they were retransformed into the conditional system (described above) to verify their phenotypes, and the complete open reading frames were sequenced by using synthetic oligonucleotides dispersed along the gene. The in vivo phenotypes of these mutants are shown in Fig. 3.

Further characterization of isolated mutations. We have used in vivo radiolabeling experiments to demonstrate that the mutations have a specific effect on protein synthesis. To determine the incubation time under nonpermissive (glucose) conditions required for depletion of eIF-4A in strain SS1-1B, cultures carrying either the wild-type *TIF2* gene or the vector were labeled at different time points. After 20 h, cells bearing a wild-type *TIF2* gene incorporated efficiently, while cells containing the vector incorporated low levels of [^{35}S]methionine. Shorter incubation times resulted in no significant difference between the strains, probably because sufficient wild-type eIF-4A protein was still present in the cell. Longer incubation times did not result in an increase of the difference in methionine incorporation. We also omitted longer incubation times to avoid secondary effects that were clearly seen in the in vitro translation system, in which longer incubation in glucose medium rendered the lysate inactive (4). We therefore labeled the cells after 20 h of growth in SD (Fig. 4).

The level of incorporation of strain SS1-1B containing the wild-type *TIF2* gene on pFL39(Δ*Cla*)-*TIF2* is shown in Fig. 4, lane 1. The negative control, SS1-1B bearing just the vector (lane 2), incorporates only about 25% as much as the

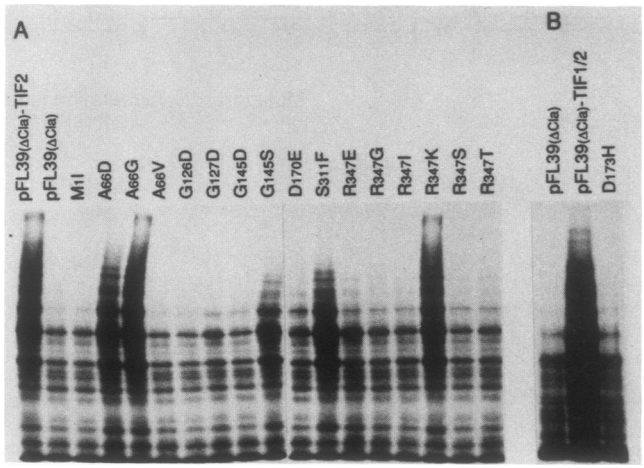

FIG. 4. In vivo protein labeling of strain SS1-1B bearing either a wild-type or a mutant *TIF* gene. Cells were labeled (as indicated in the text) with [^{35}S]methionine for 5 min and lysed with NaOH–β-mercaptoethanol, and trichloroacetic acid-precipitated proteins were analyzed by SDS-PAGE and fluorography. The mutated residues are indicated.

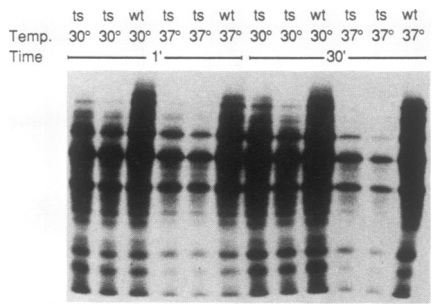

FIG. 5. In vivo protein labeling of strains SS8-3A (temperature sensitive [ts]), SS8-3B (ts), and SS8-3D (wild type [wt]). The strains were assayed in parallel under the indicated conditions. Cells were incubated for either 1 or 30 min at the indicated temperature and then labeled with [^{35}S]methionine for 5 min at the same temperature. Longer incubation times prior to labeling do not alter the result and are therefore not shown. After lysis of the cells with NaOH–β-mercaptoethanol, the proteins were trichloroacetic acid precipitated and analyzed by SDS-PAGE fluorography.

wild type, as determined by scanning of the fluorograph. The amounts of methionine incorporated by the nonviable mutants correspond approximately to the negative control (M1I, A66V, G126D, G145D, D170E, R347E, R347G, R347I, R347S, and R347T). The leaky mutants show an intermediate level of incorporation (A66D, G145S and S311F), whereas the fast-growing mutants result in wild-type levels (A66G and R347K). Mutant G127D shows [^{35}S]methionine incorporation similar to that of the negative control, in accordance with the extremely long generation time. From these results, it is clear that the relative levels of translation correlate with the respective growth rates.

Analysis of the temperature-sensitive mutation. We identified one mutation that displayed a temperature-sensitive phenotype. This mutation resides just after the ATPase A motif and results in substitution of the alanine residue at position 79 by valine (A79V). Most of the other proteins of the D-E-A-D family have a proline residue at this position.

A temperature-sensitive conditional system was constructed by sporulation of the diploid strain SS8, carrying the two plasmids pFL39-*TIF1* and pSSC120 (containing the temperature-sensitive allele on plasmid pPLI39-*TIF1*). The two cultures from spores SS8-3A and SS8-3B (carrying the temperature-sensitive mutation on plasmid pSSC120; Table 1) show slower growth at 25 and 30°C than do the two cultures from spores SS8-3C and SS8-3D, which carried the wild-type *TIF1* gene on a plasmid. Shifted to 37°C, however, these strains stop dividing immediately, as analyzed by plating for cell viability at the permissive temperature. The OD_{600} values still increase for about 20 h because of the increase in cell volume that is comparable to the increase observed in the other conditional systems. Between 12 and 33 h after the shift to the nonpermissive temperature, cell viability decreases almost 10-fold.

We have investigated the effect of this mutation on translation by performing experiments in which proteins were radiolabeled with [^{35}S]methionine in vivo. Mutant strains (SS8-3A and SS8-3B) were incubated at the nonpermissive temperature for either 1 min, 30 min, 1 h, 3 h, or 6 h and then pulsed with [^{35}S]methionine for 5 min. We observed a decrease in the efficiency of translation even after the 1-min preincubation (Fig. 5). The control strain SS8-3D, bearing a wild-type *TIF1* gene, incorporated the same amount of labeled methionine at both temperatures.

DISCUSSION

eIF-4A is a very abundant protein that performs an essential function in translation. In the yeast *S. cerevisiae*, it is encoded by two redundant genes, *TIF1* and *TIF2*, whose products are essential for cell viability and the activity of an in vitro translation system (4).

We have placed the *TIF1* gene under the control of the *GAL10-CYC1* hybrid promoter, allowing conditional expression on galactose-containing media and repression on glucose-containing media. Cells containing such a conditional system can grow on galactose, but when shifted to glucose, they stop growth after about six generations. Although we do not yet know the minimal amount of eIF-4A$_Y$ required for cell viability, a prolonged arrest in eIF-4A$_Y$ synthesis results in a complex pattern of phenotypes, including a two- to threefold increase in cell diameter, the appearance of large vacuoles, and a slow decrease in cell viability. Since eIF-4A performs an essential role in translation initiation, we believe that these phenotypes are due to secondary effects.

To learn more about the function of the eIF-4A protein, we carried out site-directed and chemical mutagenesis of the *TIF1* and *TIF2* genes. To analyze the effect of mutations in the *TIF* genes, we monitored cell viability and carried out in vivo labeling experiments. In these experiments, we incubated the cells for 20 h on glucose to reduce the level of wild-type eIF-4A$_Y$ and to study the effect of the mutant proteins on translation efficiency. In all cases analyzed, there is a good correlation between the efficiency of in vivo protein synthesis and growth rates of individual mutants.

Members of the D-E-A-D protein family contain several conserved sequences (Fig. 6). We have mutated residues within three of the main domains (ATPase A and B motifs and HRIGR motif). It has been previously noticed that an alanine residue is present in the ATPase A motif (AxxGxGKT) in most members of the D-E-A-D protein family as well as in several DNA- and RNA-interacting proteins such as DnaB, UvrD, elongation factor 1α, and transcription termination factor Rho (16, 30). Only the

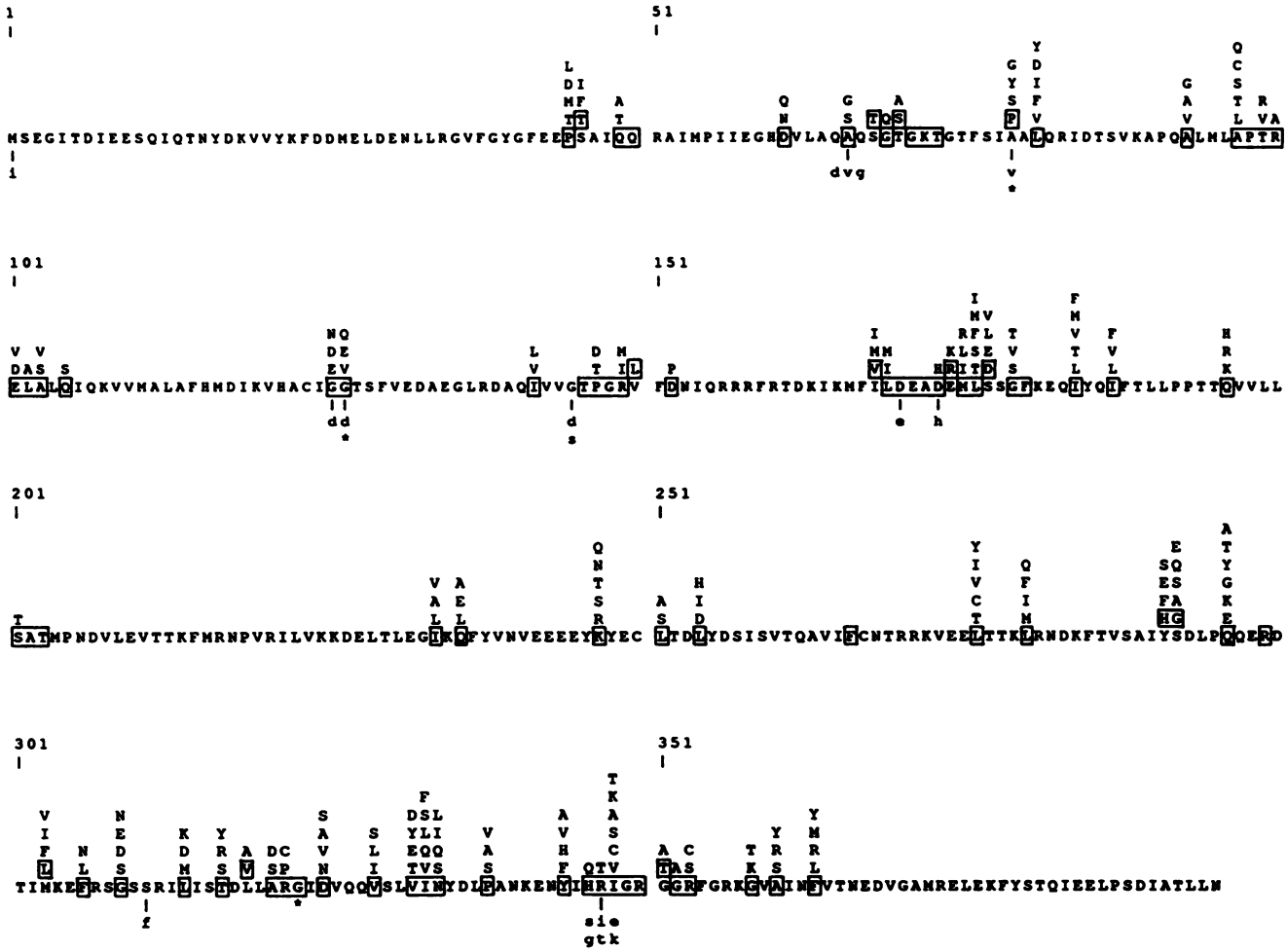

FIG. 6. Protein sequence of eIF-4A$_Y$, mutations in the *TIF* genes, and conserved residues in the D-E-A-D family. For the consensus sequence, the data from eIF-4AI (35), eIF-4AII (36), Tif1/Tif2 (32), Rm62 (14), Spp81 (26), Pl10 (29), An3 (21), Vasa (27), DbpA (24), p68 (16), SrmB (37), Prp28 (51), Prp5 (11), Mss116 (46), Spb4 (43), Dm73d (26b), Prp2 (7), Prp16 (5), and Prp22 (10) were aligned with the program PILEUP of the GCG package (version 7), and the alignment was completed by visual editing. The sequences were considered conserved if 12 or more of the 19 sequences showed the same amino acid. The predominant amino acid is boxed, and deviations from the Tif1/Tif2 sequence are indicated above the sequence. Positions of complete identity are only boxed. Below the sequence are indicated the mutations in the eIF-4A$_Y$ protein described in the text. The three asterisks indicate the positions of the *prp16-1* mutation (Y→D), the mutation in *prp28* (G→E), and the mutation in *prp22* (G→D) at positions 79, 127, and 323 of the eIF-4A$_Y$ protein, respectively. The alanine-to-valine substitution leading to a temperature-sensitive phenotype in the Tif1 protein is at position 79.

splicing proteins Prp22, Prp16, and Prp2 (5, 7, 10) have a glycine at this position, and Spb4 and Prp5 have a serine instead of alanine (11, 43). Our studies show that substitution of the alanine by valine is not viable and that substitution by aspartic acid results in reduced in vivo activity. Interestingly, the mutant containing a glycine instead of this alanine residue shows almost wild-type activity. It will be interesting to purify the mutant proteins and analyze them for ATP-binding and ATPase activities, since substitution of the lysine residue within this motif alters either ATP binding or hydrolysis (12, 42, 52).

From the combined analysis of X-ray diffraction and nuclear magnetic resonance of adenylate cyclase, it has been suggested that the aspartic acid residue of the ATPase B motif interacts with the Mg-ATP (17). We therefore mutated this residue to a glutamic acid. This resulted in a nonviable phenotype, which is not unexpected. The replacement might result in a steric hindrance, which could explain why all members of the D-E-A-D protein family possess this conserved ATPase B motif, and other ATPases involved in nucleic acid metabolism have at least the residues DE at this position (23).

Three members of the family involved in RNA splicing have the motif DEAH instead of DEAD. We therefore created a substitution of this aspartic acid by histidine in the eIF-4A$_Y$ protein. This leads to a nonviable phenotype. As the three splicing proteins that carry the DEAH motif also differ at many other conserved positions, it is reasonable to class them in a subgroup (55). It will be interesting to search for intragenic and intergenic suppressors of this D173H mutation to understand why there is a histidine instead of an aspartic acid at this position.

Mutations in the first arginine of the HRIGR motif are not viable except for the conservative substitution arginine to lysine, which results in wild-type function. This would suggest that a positive charge is essential at this position.

Interestingly, although one member of the family, the Prp5 splicing protein (11), has a threonine residue at this position, the R347T mutation abolishes eIF-4A$_Y$ function.

Most of the mutations obtained by chemical mutagenesis are located between the two ATPase motifs or very close to the HRIGR domain. The majority of the mutations occur in those positions that are highly conserved among the D-E-A-D protein family (Fig. 6). We cannot exclude, however, that the bias of a mutation in a conserved position is due to our screening procedure. In two mutants, the glycine residues 126 and 127 were changed to aspartic acid residues. The glycine 126-to-aspartic acid substitution is nonviable, whereas the glycine 127-to-aspartic acid mutation results in a very slow growth rate. Most proteins have an alanine at position 145, but glycine (eIF-4A$_Y$, mouse eIF-4A, and Spb4) or threonine (*Drosophila* Dm73d gene [26b]) residues are also found in the D-E-A-D protein family. This region is less conserved in the DEAH subgroup, whose members have a methionine or tyrosine corresponding to the glycine in the eIF-4A$_Y$ protein. We isolated two mutations at this position; substitution of the glycine by an aspartic acid results in nonviability, while its replacement by a serine results in a slow-growth phenotype. One mutation affecting a nonconserved position is the substitution of serine 311 by phenylalanine, which results in an increased cell generation time.

Although we have previously shown that the *TIF* genes code for a bona fide translation initiation factor, we cannot exclude that mutations or depletion of eIF-4A$_Y$ may cause other defects that lead to a decrease in translation.

In this report we have also characterized a temperature-sensitive mutation that results from a substitution of the alanine residue at position 79 by a valine residue. This residue is located very close to the ATPase A motif and therefore could affect ATP binding or ATPase activity. In both mouse eIF-4A proteins a serine is found at that position, whereas the other members of the D-E-A-D protein family have a proline. Cells containing only the temperature-sensitive protein can grow at 25°C, albeit at a slower rate than wild-type cells, but stop proliferating at 37°C. Protein synthesis measured by [^{35}S]methionine incorporation ceases immediately after a shift to the restrictive temperature. Prolonged incubation at the nonpermissive temperature leads to an increase in cell size and a decrease in viability. This finding shows that the mutation affects translation very quickly upon shift to the nonpermissive temperature and that the arrest in protein synthesis leads to secondary effects. The *prp16-1* mutation (5) is also situated in this region. This mutation increases the fidelity of branch point recognition in the splicing reaction.

In summary, our results demonstrate a good correlation between methionine incorporation and the generation rates of strains harboring mutant Tif proteins. These results also emphasize the importance of the domains conserved in the D-E-A-D protein family. The use of these mutants in a genetic analysis to identify suppressor mutations should provide more information on the specific protein-protein or protein-RNA interactions of the eIF-4A protein and its function as an RNA helicase.

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