Moe1 and spInt6, the Fission Yeast Homologues of Mammalian Translation Initiation Factor 3 Subunits p66 (eIF3d) and p48 (eIF3e), Respectively, Are Required for Stable Association of eIF3 Subunits*

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The Journal of Biological Chemistry 277, 2360–2367, 2002

The subunit composition of mammalian eIF3 is quite complex. Purified functional mammalian eIF3 has been reported to consist of 10 distinct polypeptides p170, p116, p110, p66, p48, p47, p44, p40, p36, and p35 (13). Likewise, wheat germ eIF3 also consists of 11 non-identical subunits (4, 5). In contrast, eIF3 purified from the budding yeast, Saccharomyces cerevisiae, contains a core complex of only five polypeptides, Rpg1p, Prt1p, Nip1p, Tif34p, and Tif39p (14–16). All five subunits of yeast eIF3 have corresponding homologues in both mammalian and wheat germ eIF3 (5). In mammalian eIF3, these homologues are p170, p116, p110, p44, and p36, respectively (5). However, the S. cerevisiae genome has no structural homologues of the mammalian eIF3 subunits p66, p48, p47, p40, and p35. This was quite surprising in view of the well accepted notion that the basic translation machinery, including the pathway of translation initiation, is highly conserved between yeast and mammals (17). The question therefore arises as to what specific functions do these additional mammalian eIF3 subunits play in eIF3 function during translation initiation. Interestingly, data base searches revealed that the genome of the fission yeast, Schizosaccharomyces pombe, contains structural homologues of four of the five mammalian eIF3 subunits, p66, p48, p47, and p40 (18, 19). These observations provide an opportunity to study the role of these four subunits in translation initiation in fission yeast. However, to date a functional eIF3 complex has not been isolated and characterized from fission yeast. Thus the subunit composition of fission yeast eIF3 is not known.

Recently, we (18, 20) and others (19, 21) have described the cloning and characterization of the S. pombe gene encoding the structural homologue of the mammalian p48 subunit (eIF3e/Int6 protein) of eIF3. S. pombe cells deleted of int6 + (Δint6) are viable and exhibit only moderate inhibition in the global rate of protein synthesis in vivo with no apparent gross defects in global translation initiation (18, 21). In addition to the int6 + gene, the S. pombe gene encoding the structural homologue of the mammalian eIF3 subunit p66/eIF3d has been cloned (22). This gene, named moel + (microtubule over extended) (22), has been shown to negatively regulate microtubule assembly/stability and chromosome segregation. However, strains deleted of moel + (Δmoe1) are also viable and show only moderate inhibition in growth rate of S. pombe cells (22). Moe1 is an evolutionarily conserved protein that shows...
41% identity and 58% similarity in amino acid sequence to the p66/eIF3d subunit of mammalian eIF3. Furthermore, mammalian eIF3-p66/eIF3d polypeptide can functionally substitute for fission yeast Moe1 in a Δmoe1 S. pombe strain (22).

In the work presented here, we have cloned and expressed the fission yeast homologue of the budding yeast eIF3 core subunit Prt1p, designated spPrt1 (the corresponding mammalian homologue is the p116/eIF3b subunit). We show that Moe1/eIF3d as well as spInt6/eIF3e physically associate with the eIF3 core subunit spPrt1 in fission yeast in a protein complex that is similar in size to multisubunit mammalian eIF3. The effects of deletion of moe1+ on global translation initiation as well as on other physiological processes were investigated. Additionally, it was observed that the presence of both Moe1 and spInt6 is required for the stable association of eIF3 subunits into a protein complex in fission yeast cells. The implications of these observations in relation to eIF3 function are discussed.

EXPERIMENTAL PROCEDURES

Strains and Media—The strains used in this study (Table I) were derived from wild-type strain 972 h+. S. pombe was grown in standard YEA (rich) and PM (minimal) media to which leucine and/or uracil were added when necessary.

Preparation of Yeast Cell Lysates, Immunoprecipitation and Western Blotting—Exponentially growing cultures of yeast cells (70 ml each) were harvested at an A\textsubscript{600} of 0.4 and washed with NET buffer (10 mM Tris-HCl, pH 8.3, 150 mM NaCl, 10 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride), then with NET buffer containing 1 M urea. Each washed pellet was then suspended in 500 \mu l of NET buffer containing 2 mM 1,4-dithiothreitol and 10% (v/v) glycerol and sonicated for 3 min. The released polypeptides in 10 \mu l of heated sample were resolved in 10% SDS-15% polyacrylamide gels. The separated polypeptides were transferred onto a polyvinylidene difluoride membrane, and analyzed by Western blotting using appropriate antibodies.

Preparation of Yeast Postribosomal Supernatant and Ribosomal Salt-wash Protein Fractions—Exponentially growing cultures of yeast cells (70 ml each) were harvested, washed with buffer B (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 15 mM MgCl\textsubscript{2}, 0.1 mM EDTA, 2.5 mM 2-mercaptoethanol), and then lysed by vortexing with glass beads in buffer B containing a mixture of protease inhibitors that included leupeptin (0.5 \mu g/ml), pepstatin A (0.7 \mu g/ml), aprotinin (2 \mu g/ml), and 0.5 mM phenylmethylsulfonyl fluoride, were lysed by vortexing with an equal volume of glass beads. Lysates were clarified by centrifugation at 12,000 \times g for 3 min. The washed beads containing bound proteins were suspended in 500 \mu l of NET buffer containing a mixture of protease inhibitors that included leupeptin (0.5 \mu g/ml), pepstatin A (0.7 \mu g/ml), aprotinin (2 \mu g/ml), and 0.5 mM phenylmethylsulfonyl fluoride, were lysed by vortexing with an equal volume of glass beads. Lysates were clarified by centrifugation at 12,000 \times g for 15 min, and immunoprecipitation was carried out at 4 °C by incubating aliquots of each cell lysate (0.5 mg of protein) with either 5 \mu g of anti-GFP mouse monoclonal antibody (Roche Molecular Biochemistry, Inc.) coupled with Protein A-agarose (Santa Cruz Biotechnology, Inc.) or 5 \mu g of anti-GFP mouse monoclonal antibody (Roche Molecular Biochemicals) that was preincubated with protein G-agarose in NET buffer. Following incubation, beads were washed 3 times with NET buffer. The washed beads containing bound proteins were suspended in 50 \mu l of 1% SDS gel loading buffer and heated in a boiling water bath for 3 min. The released polypeptides in 10 \mu l of heated sample were resolved in 10% SDS-15% polyacrylamide gels. The separated polypeptides were transferred onto a polyvinylidene difluoride membrane, and analyzed by Western blotting using appropriate antibodies.

Preparation of Yeast Postribosomal Supernatant and Ribosomal Salt-wash Protein Fractions—Exponentially growing cultures of yeast cells (70 ml each) were harvested, washed with buffer B (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 15 mM MgCl\textsubscript{2}, 0.1 mM EDTA, 2.5 mM 2-mercaptoethanol), and then lysed by vortexing with glass beads in buffer B containing a mixture of protease inhibitors as described above. After incubation at 0 °C for 30 min, the suspension was centrifuged again at 100,000 rpm for 30 min, and the supernatant containing ribosomal salt-wash proteins was dialyzed against 600 ml of NET buffer containing 70 mM NaCl and 0.5 mM phenylmethylsulfonyl fluoride for 3 h. Both the postribosomal supernatant and dialyzed ribosomal 0.5 \mu l KCl-wash proteins were analyzed by SDS-PAGE followed by Western blotting. Ribosomal 0.5 \mu l KCl-wash proteins (500 \mu g each) were also analyzed by immunoprecipitation followed by Western blotting.

Cloning and Expression of spPrt1 and spInt6 in Escherichia coli for Antibody Production—The coding sequence of the fission yeast homologue of the S. cerevisiae eIF3 subunit Prt1p/eIF3b, and the fission yeast homologue spPrt1/eIF3d were PCR amplified using appropriate primers and a fission yeast cDNA library (ZAP2, Stratagene) as a template. The PCR products were cloned in pGEX-KG vector (Amersham Bioscience, Inc.) in-frame with the glutathione S-transferase fusion tag. Both the constructs were sequenced to ensure error-free DNA synthesis. For preparation of polyclonal antibodies to the fission yeast proteins spPrt1 and spInt6, these two proteins were first expressed as glutathione S-transferase fusion proteins in bacteria. Both recombinant proteins were in the inclusion bodies in the bacterial lysates. The recombinant proteins were purified from inclusion bodies by first washing each pellet with sonication buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl\textsubscript{2}, 30 mM KCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride), then with sonication buffer containing 1 M urea. Each washed pellet was then suspended in sonication buffer containing 8 M urea and incubated overnight at 4 °C to dissolve the insoluble recombinant protein. The dissolved proteins were subjected to SDS-PAGE and the band corresponding to each fusion protein was excised and rabbit polyclonal antibodies were raised against each protein following standard protocols. Each antiserum was affinity purified against the respective antigens immobilized on polyvinylidene fluoride membranes.

Other Methods—Methods used for (a) measuring the rate of in vivo protein synthesis, (b) polysome profile analysis, and (c) detecting the association of Moe1 and other fission yeast eIF3 subunits to 40 S particles were as described (18).

RESULTS

Moe1 and spInt6 Associate with Subunits of the eIF3 Core Complex—The protein Prt1p is a bona fide subunit of budding yeast eIF3. Mutation in the PRT1 gene leads to severe defects in the translation function of eIF3 both in vivo and in vitro (23, 24). The fission yeast S. pombe genome contains the structural homologues of Prt1p, designated spPrt1, as well as of the other four eIF3 core subunits. Among these fission yeast structural homologues of the eIF3 core subunits, the only one that has been cloned and characterized so far is the protein called Sum1 (25). The gene sum1+ was originally identified in a genetic screen designed to isolate suppressors of uncontrolled mitosis (25). Sum1 is the fission yeast structural homologue of the mammalian eIF3 core subunit p36/eIF3i (the corresponding budding yeast homologue is Tif34p (5, 26)). Additionally, in this work we have cloned and expressed in E. coli the coding sequence of the fission yeast homologue of the budding yeast eIF3

| Table I | Fission yeast strains used in this study |
|---|---|
| Strain | Genotype | Ref. or source |
| 972 | h+ | This work |
| AE 399 | h+ | This work |
| SP 6 | h+ | This work |
| UM 201 | h+ | Ref. 18 |
| UM 210 | h+ | This work |
| UM 209 | h+ | This work |
| UM 211 | h+ | This work |
| UM 105 | h+ | This work |
| UM 113 | h+ | This work |
| UM 114 | h+ | This work |
| UM 109 | h+ | This work |
| UM 110 | h+ | This work |

The designations in Table I follow standard genetic nomenclature for the budding yeast S. pombe. All GFP-Sum1 expressing strains used in this study were derived from S. pombe (h- ade6–210 sum1::ura4+ leu1::(GFP-sum1+) (25).

This work
core subunit Prt1p, and raised antibodies against the purified protein (see “Experimental Procedures”). The availability of antibodies to spPrt1 and Sum1 has allowed us to perform coimmunoprecipitation experiments to investigate whether spPrt1 and Moe1, the two non-core eIF3 subunits that have no homologues in the budding yeast genome, associate with these two core eIF3 subunits of fission yeast. Our assumption is that association of these non-core subunits with the core subunits spPrt1 and Sum1 represents association with the multisubunit fission yeast eIF3 complex.

We constructed two fission yeast strains, designated UM 210 and UM 201 (Table 1). The strain UM 210 expresses both GFP epitope-tagged Sum1 (GFP-Sum1) and Myc epitope-tagged spInt6 (Myc-spInt6), whereas the strain UM 201 expresses only Myc-spInt6. Cell-free extracts from UM 210 and UM 201 were prepared and subjected to immunoprecipitation using anti-GFP (Fig. IA) or anti-Myc (Fig. 1B) antibody. The resulting immunocomplexes were examined for the presence of the two eIF3 core subunits spPrt1 and GFP-Sum1 as well as Moe1 and Myc-spInt6 by Western blot analysis. The anti-GFP antibody immunoprecipitated GFP-Sum1 from cell-free extracts of UM 210 as expected (Fig. IA, compare lanes 1 and 3). The precipitated immunocomplex contained, in addition to GFP-Sum1, Moe1, and Myc-spInt6 polypeptides as well as spPrt1, another core subunit of eIF3 (Fig. 1A, compare lanes 1 and 3). The specificity of coimmunoprecipitation by anti-GFP antibody was demonstrated by our observation that anti-GFP antibody did not immunoprecipitate spPrt1, Moe1, or Myc-spInt6 unless GFP-Sum1 was present in the lysate (Fig. 1A, compare lanes 2 and 4). Similar results were obtained when extracts of UM 210 and UM 201, both of which expressed Myc-spInt6, were immunoprecipitated with anti-Myc antibody. Under these conditions, the immunocomplex precipitated from cell extracts derived from either strain contained, in addition to Myc-spInt6, spPrt1, and Moe1 polypeptides (Fig. 1B, lanes 3 and 4). Furthermore, the immunocomplex precipitated from extracts of UM 210 also contained GFP-Sum1 polypeptide (Fig. 1B, lane 2). These results show that in fission yeast, both Moe1 and spInt6 polypeptides are present in a complex with at least two eIF3 core subunits, spPrt1 and GFP-Sum1.

Moe1 Associates with 40 S Ribosomal Particles as a Constituent of Multisubunit Fission Yeast eIF3 Protein Complex—An important property of mammalian eIF3 is that in cell lysates, eIF3 is found almost exclusively bound to 40 S ribosomal particles (7) from which the initiation factor can be dissociated by treatment with 0.5 M KCl-containing buffer. We examined whether Moe1, like its mammalian homologue p66, can associate with 40 S ribosomal particles as a component of fission yeast eIF3. For this purpose, cell-free extracts of wild-type fission yeast SP6 were subjected to sucrose gradient centrifugation and the gradient fractions were examined for the presence of Moe1, spPrt1, and spInt6 by Western blot analysis. A 43 S preinitiation complex (40 S eIF3mRNA:Met-tRNAf:elF2-GTP) formed in vitro (27) and analyzed in a parallel gradient tube, served as a marker for the 40 S particles while in another parallel gradient, a preformed 80 S initiation complex (80 S: AUG-Met-tRNAf) was analyzed to indicate the lower boundary of the polysomes (Fig. 2A). Western blot analysis of each gradient fraction showed that Moe1, spPrt1, and spInt6 sedimented at a position where the 43 S preinitiation complex also sedimented (Fig. 2B, top three panels). Further confirmation that spPrt1, Moe1, and spInt6 sedimented with the 40 S particles is derived from the observation that the gradient fractions containing these polypeptides specifically included only 18 S rRNA, a known constituent of 40 S ribosomal subunits (Fig. 2B, last panel). These observations indicate that Moe1 and spInt6 associate with 40 S particles in fission yeast. It should be noted that in addition to its association with 40 S particles, a fraction of the eIF3 subunits also sedimented bound to 80 S ribosomes and/or polysomes indicating that a small fraction of eIF3 is associated with 80 S ribosomes and/or polysomes. Additionally, the presence of eIF3 subunits in the lighter fractions suggests that in cell-free extracts, a fraction of eIF3 subunits may be present as free polypeptides.

We also investigated whether both Moe1 and spInt6 associate with the ribosomal particles as constituents of the eIF3 protein complex. For this purpose, ribosomes were isolated from extracts of UM 210 and then treated with 0.5 M KCl to dissociate the bound proteins. The ribosomal 0.5 M KCl-wash proteins and the postribosomal supernatant protein fractions were then analyzed by Western blotting using antibodies specific for each indicated eIF3 subunit. For each immunoprecipitation, 100 μg of cell-free extracts were also directly subjected to Western blot analysis as loading controls, designated “Input.” Panel C, ribosomal salt-wash and postribosomal supernatant protein fractions were prepared from wild-type SP6 and UM 210 cells as described under “Experimental Procedures.” Both the postribosomal supernatant (lane 1) and ribosomal salt-wash (lane 2) protein fractions (100 μg each), prepared from UM 210, were analyzed by SDS-PAGE followed by Western blotting using antibodies specific for each indicated eIF3 subunit. In addition, 500 μg of ribosomal salt-wash protein fraction from both SP6 (lane 3) and UM 210 (lane 4) were subjected to immunoprecipitation using anti-Myc monoclonal antibody. The immunocomplexes were analyzed by SDS-PAGE followed by Western blotting using antibodies specific for each indicated eIF3 subunit. The asterisk (*) denotes a nonspecific cross-reacting polypeptide observed with anti-GFP rabbit antibodies.

**Fig. 1.** Association of Moe1 and spInt6 with eIF3 core subunits. Immunoprecipitation was carried out with anti-GFP (panel A) or anti-Myc (panel B) monoclonal antibody from about 500 μg of cell-free extracts of UM 210 (lanes 1 and 3) or UM 201 (lanes 2 and 4), as described under “Experimental Procedures.” Following immunoprecipitation, each immunocomplex (designated as “Immunocomplex”) was resuspended in 50 μl of SDS loading buffer and boiled. Aliquots (10 μl each) were analyzed by SDS-PAGE followed by immunoblotting using antibodies specific for each indicated eIF3 subunit. For each immunoprecipitation, 100 μg of cell-free extracts were also directly subjected to Western blot analysis as loading controls, designated “Input.” Panel C, ribosomal salt-wash and postribosomal supernatant protein fractions were prepared from wild-type SP6 and UM 210 cells as described under “Experimental Procedures.”
C, lanes 1

(Fig. 1 was observed for the eIF3 core subunits, spPrt1 and GFP-Sum1 fraction (Fig. 1C, compare these two proteins was found in the postribosomal supernatant during fractionation by centrifugation in 15% glycerol gradient, 11 ml and centrifuged in a SW41 rotor at 40,000 rpm for 22 h. In two parallel tubes, purified rabbit reticulocyte eIF3 (8) and molecular weight standards were analyzed. Following centrifugation, fractions of 0.5 ml were collected from the bottom of each tube. Proteins in the gradient fractions were precipitated in the presence of 16% trichloroacetic acid, resuspended in SDS-loading buffer, and subjected to SDS-PAGE. The gels containing purified rabbit reticulocyte eIF3 were stained with Coomassie Brilliant Blue to establish the position of reticulocyte eIF3 (Panel B). Proteins present in gels containing fission yeast ribosomal salt-wash protein fractions were transferred to polyvinylidene fluoride membranes which were then subjected to Western blotting with the indicated antibodies (The four panels in A. It should be noted that in Panel A, all 23 fractions collected were analyzed by Western blotting. However, only the results from fractions 6 through 23 are shown since the first five fractions did not have any immunoreactive band. In Panel B, only fractions 7 through 20 were analyzed since the sedimentation profile of purified mammalian eIF3 was known previously.

salt-wash protein fraction whereas only a minor fraction of these two proteins was found in the postribosomal supernatant fraction (Fig. 1C, compare lanes 1 and 2). A similar distribution was observed for the eIF3 core subunits, spPrt1 and GFP-Sum1 (Fig. 1C, lanes 1 and 2). Furthermore, when the ribosomal salt-wash proteins were subjected to immunoprecipitation using anti-Myc antibody, the precipitated immunocomplex contained, in addition to Myc-splnt6, spPrt1, GFP-Sum1 as well as Moe1 (Fig. 1C, lane 4). When similar immunoprecipitation was carried out using ribosomal salt-wash proteins derived from lysates of wild-type SP6 fission yeast cells, which did not express any Myc-tagged polypeptide, neither Moe1 nor spPrt1 was immunoprecipitated (Fig. 1C, lane 3). These results suggest that Moe1 and Myc-splnt6 were bound to ribosomes as components of the eIF3 protein complex.

Further confirmation for this conclusion came from the observations that when the ribosomal 0.5 M KCl-wash protein fraction from wild-type (UM 210) cells were subjected to size fractionation by centrifugation in 15–40% glycerol gradients, a significant fraction of spPrt1, GFP-Sum1, Moe1, and Myc-splnt6 sedimented at a position corresponding to an apparent Mr of about 400,000 (Fig. 3A). Purified functional mammalian eIF3, a protein complex of 10 subunits, run in a parallel gradient, sedimented at a similar position (Fig. 3B). These results indicate that spPrt1, GFP-Sum1, Moe1, and Myc-splnt6 are part of a higher molecular weight protein complex, which presumably represents the multisubunit fission yeast eIF3 consisting of the core and non-core subunits. It should be noted that in addition to sedimenting as a higher molecular weight protein complex, a small fraction of the eIF3 subunits also sedimented near the top of the gradient. Presumably they represent free eIF3 polypeptides.

Moe1 Is Not Essential for Global Translation Initiation—Our observation that Moe1 is a constituent of fission yeast eIF3 protein complex prompted us to examine the function of Moe1 in translation initiation using a Δmoe1 fission yeast strain. Exponentially growing cultures of a Δmoe1 strain (UM 108) and a wild-type strain (SP6) were pulse-labeled with [35S]methionine and the rate of protein synthesis was measured by incorporation of [35S]methionine into polypeptide chains over a 30-min period. The rate of protein synthesis in Δmoe1 cells was about 30–40% slower than in wild-type cells (Fig. 4, panel A). This slower rate of methionine incorporation into cellular proteins in UM 108 cells is in keeping with the reduced growth rate of these cells when compared with wild-type strains (22). Additionally, we examined the polysome-ribosome profile of exponentially growing cultures of Δmoe1 (UM 108) and control wild-type SP6 cells by subjecting cell-free extracts of these strains to sucrose gradient centrifugation (Fig. 4, panel B). The polyribosome content of both strains showed almost similar
growth medium (Fig. 5). Furthermore, like Δint6 cells (18), Δmoe1 cells grew slowly in minimal media (data not shown).

We previously observed (18) that upon conjugation, Δint6 cells often produce asci with less than four spores. Likewise, when UM 113 (h+, Δmoe1) and UM 114 (h-, Δmoe1) were crossed, only about 40% of the ascis formed after ~48 h had a complete tetrad set (Fig. 6 and Table II). Of the remaining asci, ~40% had no visible spores while ~20% contained less than four spores (Fig. 6, see also Table II). In contrast, when two wild-type fission yeast strains were crossed for the same time period, nearly 90% of the ascis were normal (Fig. 6 and Table II). Likewise, the cross between the Δmoe1 strain, UM 114 (h+) and the wild-type strain 972 (h-) also behaved like crosses between two wild-type strains (Fig. 6, Table II). These results suggested that Moe1, as with spInt6, is required for meiosis and/or sporulation in fission yeast cells. Furthermore, the presence of Moe1 or spInt6 in one of the two conjugating partners is sufficient for successful sporulation following conjugation.

We also examined whether Moe1 and spInt6 participate in the same or parallel pathways to promote sporulation in fission yeast. As shown in Table II, crosses between either Δint6 or Δmoe1 cells alone were as defective in sporulation as were crosses between Δint6 and Δmoe1 cells indicating that Moe1 and spInt6 may function in the same or parallel pathways that support sporulation in S. pombe. Further confirmation that spInt6 and Moe1 function in the same pathway came from the observation that overexpression of spInt6 in Δmoe1 cells did not rescue the growth defects of these cells (data not shown). Similar conclusions were also reached in a previous study (20). Moe1 and spInt6 Are Required for Stable Association of eIF3 Subunits in a Protein Complex—In view of our findings that both Δint6 and Δmoe1 cells show similar defects in translation and other biological processes, we examined whether the incorporation of both polypeptides into the eIF3 protein complex is interdependent. For this purpose, we generated a Δint6ΔspInt6 strain (UM 211) and a Δmoe1ΔspInt6 strain (UM 209) fission yeast strain. When cell-free extracts of UM 209 (Δmoe1ΔspInt6) were subjected to immunoprecipitation with anti-Myc antibody, the precipitated immunocomplex contained Myc-spInt6 (Fig. 7B, lane 4). However, when the immunocomplex was examined for the presence of either GFP-Sum1 or spPrt1 by Western blot analysis, neither of these polypeptides were communoprecipitated with Myc-spInt6 (Fig. 7B, compare lanes 2 and 4). Likewise, when these cell extracts were subjected to immunoprecipitation with anti-GFP antibody, spPrt1 was also not communoprecipitated with GFP-Sum1 (Fig. 7A, compare lanes 3 and 4 with lanes 1 and 2, respectively). In contrast, when similar immunoprecipitation experiments were carried out with wild-type extracts, all of the eIF3 polypeptides

FIG. 5. Effect of caffeine on Δmoe1 cells and suppression of caffeine sensitivity by sorbitol. Strains 972 (WT, h+) and UM 114 (Δmoe1, h-) were grown on YEAU plates (YEAU) or YEAU plates containing 12.5 mM caffeine (YEAU + Caffeine) or YEAU plates containing 12.5 mM caffeine and 1.2 M sorbitol (YEAU + Caffeine + Sorbitol). The plates were incubated at 32 °C for 16 h.

FIG. 4. Δmoe1 cells are not defective in global translation initiation. Panel A, in vivo incorporation of [35S]methionine in wild-type and Δmoe1 fission yeast cells. Total incorporation of [35S]methionine into proteins in a wild-type or Δmoe1 strain was determined. Panel B, analysis of polysomes. Cell lysates were prepared from exponentially growing cultures of a wild-type SP6 strain (WT) or UM 108 (Δmoe1) strain in YEAM medium at 32 °C and subjected to 7–47% (w/v) sucrose gradient as described previously (18). Each gradient was fractionated in an ISCO gradient fractionator and the absorbance profile at 254 nm was analyzed using an ISCO UA-5 absorbance monitor.

Profiles. If the presence of Moe1 in eIF3 were required for global translation initiation, its absence in fission yeast cells would have caused extensive breakdown of polysomes with simultaneous increase in the pool of free 80 S ribosomes and free 60 S and 40 S ribosomal subunits as was observed previously when eIF5, an essential translation initiation factor, was either depleted from (28) or inactivated in budding yeast cells (29). These results indicate that Δmoe1 cells are not defective in the initiation of translation of a majority of cellular mRNAs. However, since Δmoe1 cells show a 30–40% slower rate of [35S]methionine incorporation, the possibility exists that Moe1 is required for either the optimal rate of translation of all mRNAs or for the translation of a specific subset of mRNAs.

Δmoe1 Fission Yeast Cells Show Similar Phenotypes as Δint6 Cells—It has been reported (20) that spln6 and Moe1 can form a complex in vitro. This observation, coupled with the above results that deletion of either moe1” or int6” (18) affects translation in fission yeast similarly, prompted us to examine whether the Δmoe1 strain shows similar phenotypic properties as those previously reported for Δint6 strains (18–21). In the presence of 12.5 mM caffeine, Δmoe1 cells failed to grow (Fig. 5) and lysed in caffeine-containing media (data not shown). As observed with Δint6 cells (18), the caffeine-mediated lysis of Δmoe1 cells was prevented by the presence of sorbitol in the
examined were communoprecipitated (see Fig. 1). These results were surprising in view of the well established fact that functional eIF3 is a multisubunit protein complex. Furthermore, in both Δmoe1 and Δint6 fission yeast cells, global initiation of translation is not drastically affected (Refs. 18 and 21, Table II).

Table II

| h− | h+ | % Perfect tetrad | % Incomplete tetrad | % No spore |
|----|----|----------------|-------------------|-----------|
| WT | WT | 89.4          | 0.625             | 10.0      |
| Δint6 | Δint6 | 39.8          | 21.5              | 38.7      |
| Δmoe1 | Δmoe1 | 41.5          | 19.9              | 38.6      |
| Δint6 | Δmoe1 | 57.5          | 22.8              | 19.8      |
| Δmoe1 | Δint6 | 48.3          | 20.7              | 31.0      |
| WT | Δmoe1 | 85.9          | 2.9               | 11.2      |
| WT | Δint6 | 84.1          | 3.5               | 12.4      |

We examined whether all the constituents of fission yeast eIF3 were still associated with ribosomes in Δmoe1 and Δint6 cells. For this purpose, ribosomes were isolated from cell-free extracts of wild-type (UM 210), Δmoe1 (UM 209), and Δint6 (UM 211) cells, and ribosome-associated proteins were isolated by treatment with 0.5 M KCl and then examined for the presence of eIF3 subunits by Western blotting. As shown in Fig. 8A (lanes 2), a major fraction of all the eIF3 subunits examined were associated with ribosomes both in wild-type and in Δmoe1 and Δint6 cells. However, in contrast to wild-type cells where all the eIF3 subunits present in the ribosomal salt-wash fraction communoprecipitated with GFP-Sum1, both in Δmoe1 and Δint6 cells, no eIF3 subunits examined including the core subunit spPrt1 communoprecipitated with GFP-Sum1 (Fig. 8B, lanes 2). These results suggest that even in Δmoe1 and Δint6 cells, a significant fraction of functional eIF3 is still associated with the 40 S particles to carry out normal levels of global protein synthesis. However, when the bound proteins were dissociated from ribosomes by 0.5 M KCl treatment, the eIF3 subunits were no longer associated in a protein complex.

Thus, it appears that stable association of eIF3 subunits to form a multisubunit protein complex in the absence of ribosomes requires Moe1 and spInt6. Additional confirmation for this conclusion came from our observation that when ribosomal 0.5 M KCl-wash protein fraction from Δmoe1 cells was subjected to size fractionation under conditions similar to those described in the legend to Fig. 3, none of the eIF3 subunits including the core subunit spPrt1p sedimented as a high molecular weight protein complex. Rather, these subunits sedimented near the top of the gradient presumably as free polypeptides (data not shown).
Additional conclusions that can be derived from the experiments presented in Fig. 8 are as follows. First, the recruitment of Moe1 to 40 S particles is independent of Spt6 (Fig. 8A, lane 2, of Δmoe1 cells) and vice versa (Fig. 8A, lane 2, of Δmoe1 cells). Second, when GFP-Sum1 was immunoprecipitated from the postribosomal supernatant, only a minor fraction of spPrt1, and no detectable levels of spInt6 or Moe1 coimmunoprecipitated with GFP-Sum1 (Fig. 8A, compare lanes 1 of panels A and B). These results indicate that the eIF3 subunits present in the postribosomal supernatant of even wild-type cells are free subunits.

It should be noted that though the eIF3 subunits in Δmoe1 and Δint6 cells were bound to 40 S particles, similar to the situation in wild-type cells, the association between the bound subunits in the deleted strains must be very weak since anti-GFP antibodies failed to coimmunoprecipitate any of the other eIF3 subunits with GFP-Sum1 even from cell-free extracts of these strains (see Fig. 7).

**DISCUSSION**

Our understanding of the mechanism of translation initiation has been greatly facilitated by complementing in vitro biochemical studies using purified mammalian translation initiation factors with molecular genetic analysis in the budding yeast, *S. cerevisiae*. This has become possible because the basic translation machinery, including the pathway of translation initiation, is highly conserved between yeast and mammals. In fact, most of the mammalian translation initiation factors have been shown to substitute for the corresponding yeast factors in vivo (3). The only exception is the multisubunit initiation factor eIF3. The genome of budding yeast does not contain any genes related to mammalian eIF3 subunit p66, as well as other non-core subunits p48 (Int6), p47, p40, and p35. In contrast, except p35, all these mammalian eIF3 subunits as well as the five core subunits which have orthologs in *S. pombe* appear to be conserved in *S. pombe*. Thus, *S. pombe* has the potential to be valuable for a more extensive investigation of the role of the individual subunits of eIF3.

Recent demonstration (22) that the fission yeast gene moe1+ is the *S. pombe* homologue of human eIF3d (p66) subunit has prompted us to characterize the properties of Moe1 protein, its association with the fission yeast eIF3 core subunits, its requirement for translation initiation, and the apparent phenotypes exhibited by Δmoe1 cells. We show that in cell-free extracts of *S. pombe*, Moe1 can be coimmunoprecipitated with two eIF3 core subunits, spPrt1 (eIF3b) and Sum1 (eIF3i) as well as another non-core subunit, spInt6 (eIF3e) (Fig. 1). Furthermore, a major fraction of Moe1 cosediments with the 40 S ribosomal subunit as a component of a protein complex that also contains spPrt1, Sum1, and spInt6. When this protein complex is isolated from ribosomes by 0.5 M KCl treatment and subjected to size fractionation by glycerol gradient centrifugation, all of these four subunits cosedimented at a position similar to that of multisubunit mammalian eIF3 (Fig. 3). These observations suggest that the fission yeast Moe1 either associates with the core subunits of eIF3 by protein-protein interaction or the polypeptide is a bona fide subunit of eIF3. However, despite the evidence that Moe1 associates with 40 S ribosomal particles as a constituent of eIF3 protein complex, fission yeast cells lacking moe1+ (Δmoe1) support global protein synthesis exhibiting polysome profiles similar to wild-type cells. It should be noted that the overall rate of protein synthesis, measured by [35S]methionine incorporation, is reduced moderately in Δmoe1 cells compared with wild-type cells. The possibility exists that Moe1 is required for translation of a subset of mRNAs or for optimal efficiency of the rate of translation initiation of all mRNAs.

The phenotypes of Δmoe1 cells mimic those of Δint6 cells (18–20). We show that like Δint6 cells, Δmoe1 cells are also hypersensitive to caffeine and this hypersensitivity can be suppressed by addition of sorbitol to the media. This suggests that Δmoe1 cells lose their viability due to osmotic stress caused by the drug. Δmoe1 cells also exhibit a slow growth rate and a defect in meiosis following conjugation (Fig. 6 and Table II). The similarities in biochemical properties and apparent phenotypes of Δint6 and Δmoe1 cells suggest that these two proteins converge on the same cellular target, presumably the eIF3 protein complex, to promote multiple biological functions. In this context, it should be noted that Moe1 has been shown to interact directly with spInt6 (20). When either protein is absent, the other becomes mislocalized and its protein level is decreased (20). We have also observed in this study that in the absence of spInt6, the level of Moe1 in UM 211 (Δint6) was decreased significantly (compare lanes 1 and 2 of panels A and B of Fig. 1 with lane 1 of panels A and B of Fig. 7).

The question arises about the functional significance of the association of spInt6 and Moe1 non-core subunits with the eIF3 core protein complex. This is particularly relevant in view of the observation that in the absence of either polypeptide, global translation is not drastically affected, and cells deleted of either gene are viable. Some insight into the importance of the association of both spInt6 and Moe1 with eIF3 core subunits emerges from coimmunoprecipitation experiments with cell extracts derived from Δmoe1 and Δint6 cells (Figs. 7 and 8). We observed that a major fraction of the eIF3 core subunits spPrt1 and GFP-Sum1, as well as the non-core subunits spInt6 or Moe1, are associated with ribosomes in wild-type and in both Δmoe1 and Δint6 cells. However, once bound eIF3 was dissociated from ribosomes of the mutant cells by treatment with 0.5 M KCl, neither the core subunits spPrt1 and Sum1 nor spInt6 or Moe1 were associated with one another in a protein complex. In contrast, all the eIF3 subunits present in the ribosomal salt-wash protein fraction derived from wild-type cells were associated with one another in a protein complex. These observations suggest that both Moe1 and spInt6 are required for the stable association of eIF3 subunits to form the multisubunit protein complex. It is likely that in the absence of either Moe1 or spInt6, the rate of association of eIF3 subunits to form the eIF3 protein complex is reduced resulting in a kinetic delay in the recruitment of eIF3 protein complex to 40 S ribosomal particles. This may also explain why in Δmoe1 and Δint6 cells, the presumptive rate of translation as measured by [35S]methionine incorporation, is slower than that in a wild-type isogenic strain.

An important question that remains unanswered in the present study is whether the phenotypes observed for Δmoe1 and Δint6 cells, e.g. hypersensitivity to caffeine, defective spore formation etc., are due to the absence of these proteins from the fission yeast eIF3 protein complex. Since formation of the 40 S eIF3 complex is a stringent prerequisite for translation initiation, it is likely that the formation of 40 S eIF3 complex is delayed in the absence of either spInt6 or Moe1. This can reduce the efficiency of the cellular translation machinery below a critical threshold that specifically affects translation of some rare messages whose protein products are involved directly in the biological processes that are affected in these deleted strains. The possibility also exists that in addition to their roles as components of eIF3 in translation initiation, these proteins have other roles unrelated to translation initiation (20, 22, 30) and the phenotypes observed for Δmoe1 or Δint6 cells result from defects in those biological functions. Further work is necessary to answer these questions.

**Acknowledgments**—We are grateful to Dr. Tim Humphrey, MRC Radiation and Genome Stability Unit, UK, for the *S. pombe* strain.
(h−ade6−210 sum1−::ura3−1::leu1−1::GFP-sum1−). We also thank Dr. C. R. Chen of the Chang laboratory of New York University for his assistance in the preparation of anti-Moe1 antibodies, and we are indebted to Dr. Jerard Hurwitz, Memorial Sloan-Kettering Cancer Center, New York, for critically reading the manuscript.

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Moe1 and spInt6, the Fission Yeast Homologues of Mammalian Translation Initiation Factor 3 Subunits p66 (eIF3d) and p48 (eIF3e), Respectively, Are Required for Stable Association of eIF3 Subunits
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J. Biol. Chem. 2002, 277:2360-2367.
doi: 10.1074/jbc.M107790200 originally published online November 8, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107790200

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