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Fission Yeast Int6 Is Not Essential for Global Translation Initiation, but Deletion of *int6*$^+$ Causes Hypersensitivity to Caffeine and Affects Spore Formation

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Mammalian INT6 protein has been considered to be a subunit of the eukaryotic translation initiation factor, eIF3. The Int6 locus is also known as a common integration site of *m*ouse *m*ammary *t*umor *v*irus (MMTV). However, the function of Int6 in translation initiation and the mechanism of Int6-mediated tumor induction are yet to be explored. In this study, the fission yeast, *Schizosaccharomyces pombe*, *int6*$^+$, which is 43% identical to the mammalian counterpart, was deleted. Despite the evidence that the majority of Int6 protein was associated with 40S particles in this organism, strains lacking *int6*$^+$ (Δ*int6*) were viable and showed only moderate inhibition in the rate of in vivo global protein synthesis. Polysome profile analysis showed no apparent defects in translation initiation. Δ*int6* exhibited a hypersensitivity to caffeine, which could be suppressed by the addition of sorbitol to the growth medium. This and other phenotypes would imply that *int6*$^+$ is required for the integrity of cell membrane. In meiosis, Δ*int6* produced incomplete tetrads frequently. High dosage expression of a truncated mutant of *int6*$^+$ conferred a hypersensitivity to caffeine, but did not cause the defect in meiosis. A possible link between the function of *int6*$^+$ and the Δ*int6*-phenotypes is discussed.

INTRODUCTION

Initiation of translation in eukaryotic cells requires the participation of GTP and a large number of specific proteins called *e*ukaryotic translation *i*nitiation *f*actors (eIFs). Among the initiation factors, eIF3 is the most complex initiation factor known with respect to both its function and its subunit composition. In vitro, the factor binds to the 40S ribosomal subunit and the 40S-bound eIF3 plays an essential role in the eIF1A-dependent transfer of initiator Met-tRNA$_f$ (as Met-tRNA$_f$·eIF2·GTP ternary complex) to 40S subunits to form the 40S-preinitiation complex (40S·eIF3·Met-tRNA$_f$·eIF2·GTP) (Chaudhuri *et al.*, 1999). The presence of eIF3 bound to the 40S preinitiation complex is also a stringent prerequisite for the 40S preinitiation complex to bind at the 5′-capped end of mRNA as well as for the subsequent scanning of the mRNA by the 40S preinitiation complex to locate the initiation AUG codon of the mRNA to form the 40S initiation complex (40S·eIF3·mRNA·Met-tRNA$_f$·eIF2·GTP) (Merrick and Hershey, 1996; Kozak, 1999). The precise mechanism by which eIF3 functions in each of the above steps is yet to be defined.

There is considerable uncertainty regarding the subunit composition of functional eIF3. The factor has been isolated in several laboratories from a variety of eukaryotic sources based on an assay that measured its ability to stimulate mRNA translation in a protein synthesizing system reconstituted with purified proteins (Benne and Hershey, 1976; Safer *et al.*, 1976; Schreier *et al.*, 1977; Merrick, 1979; Spremulli *et al.*, 1979; Checkley *et al.*, 1981; Brown-Luedi *et al.*, 1982; Seal *et al.*, 1983). Mammalian eIF3, purified in this way, was reported to consist of ten major polypeptides, p170, p116, p110, p66, p48, p47, p44, p40, p36,and p35 (Hershey *et al.*, 1996). In contrast to mammalian eIF3, eIF3 purified from *Saccharomyces cerevisiae* on the basis of an assay that measures AUG-dependent methionyl-puromycin synthesis consisted of eight major polypeptides of apparent molecular masses, 135, 90, 62, 39, 33, 29, 21, and 16 kDa (Naranda *et al.*, 1994). However, when budding yeast eIF3 was purified based on a direct assay for the presence of Prt1p, a known subunit of yeast eIF3, it was observed that Prt1p copurified with only four other polypeptides (Danaie *et al.*, 1995; Phan *et al.*, 1998). This five-subunit core complex,

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consisting of Rpg1p (also designated Tif32p), Prt1p, Nip1p, Tif34p, and Tif35p subunits, stimulated the transfer of Met-tRNA$_f$ to 40S ribosomal subunits nearly 10-fold (Danaie *et al.*, 1995; Phan *et al.*, 1998).

Comparison of the predicted amino acid sequence of each of the 10 mammalian eIF3 subunits with the derived protein sequences in the yeast *S. cerevisiae* genomic data base revealed that all the five subunits of yeast eIF3 core complex (Rpg1p, Prt1p, Nip1p, Tif34p, and Tif35p) have corresponding homologues in mammalian eIF3, which are p170, p116, p110, p36, and p44, respectively (Asano *et al.*, 1998). However, the other mammalian eIF3 subunits, p66, p48, p47, p40, and p35 have no structural homologues in *S. cerevisiae*. This was somewhat 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 (Donahue and Huang, 1997).

Among the mammalian eIF3 subunits that have no structural homologues in budding yeast, the p48 subunit is of particular interest. Sequence analysis of the p48-cDNA showed that p48 is identical to the mouse protein INT6 (Asano *et al.*, 1997). The genomic locus of Int6 has been a frequent integration site for *m*ouse *m*ammary *t*umor *v*irus (MMTV) (Marchetti *et al.*, 1995). This integration induces formation of mammary tumors in mice although the mechanism of tumor formation is not clear. The key question from the point of view of translation is what specific role p48 (INT6), as a subunit of mammalian eIF3, performs in translation initiation in mammalian cells. Interestingly, database searches revealed that p48 has a structural homologue in the fission yeast *Schizosaccharomyces pombe*. This observation provided a unique opportunity to study the role of p48 (Int6) in translation initiation in *S. pombe*.

In this paper, we describe the cloning and characterization of the *S. pombe* gene encoding Int6 protein. Deletion of *int6*$^+$ (Δ*int6*) is viable and exhibits only moderate inhibition in the rate of in vivo protein synthesis, with no apparent defects in translation initiation. Δ*int6* as well as expression of a C-terminally truncated version of Int6 cause phenotypes suggestive of a defect in the maintenance of cell wall/membrane integrity.

MATERIALS AND METHODS

Strains and Media

All strains used in this study were derived from wild-type strains. *S. pombe* was grown in standard YEA and PM media to which leucine and/or uracil were added when necessary.

Cloning of int6$^+$ and Plasmid Construction

BLAST (*B*asic *L*ocal *A*lignment *S*earch *T*ool) search of the web-based genome database (www.Sanger.ac.uk/BLAST) of the Sanger Centre (Cambridge, United Kingdom) database with human Int6 sequence revealed that the *S. pombe* homologue of human Int6 is present in cosmid No. 646. A 3.5-kb *Xho*I/*Spe*I fragment containing *int6*$^+$ was obtained from cosmid No. 646 and cloned into pBluescript SK (-) to generate the plasmid pINT6. The coding sequence of Int6 cDNA was amplified by PCR from an *S. pombe* cDNA library, using *pfu* DNA polymerase (Stratagene. L Jolla, CA) and two primer sequences as follows: N-terminus, 5′-d GGCACTCGAGCATATGGGGTCCGAGCTTAAGAG-3′ having a *Xho*I/*Nde*I overhang; C-terminus, 5′-dAGCTGAATTCGGATCCCTAAACAGTAGCATGCTT-3′ having a *Bam*HI/*Eco*RI overhang. The PCR product was digested with *Nde*I and *Bam*HI, was cloned into the same sites of pREP41 to generate the thiamine-repressible pREP41-INT6 expression plasmid, and was sequenced to ensure error-free DNA synthesis. To express the C-terminally truncated 50 amino acid shorter version of Int6 protein, the same N-terminal primer was used in the PCR reaction, while the other primer used for C-terminus was 5′-d AGCTGAATTCGGATCCCTACTTAGCTTTAAACCCAAATTG -3′. The PCR product was digested with *Nde*I and *Bam*HI and was cloned in pREP41 to generate a thiamine-repressible pREP41-CΔ50 INT6 expression plasmid. Wild-type cells were transformed following standard procedures.

Gene Disruption

An *Sph*I site was generated at a *Bam*HI site on pINT6, which is 4 bp downstream of the first ATG initiation codon of the putative *int6*$^+$ open reading frame. The modified plasmid pINT6–1 contained two *Sph*I sites in the open reading frame of *int6*$^+$, one generated at the *Bam*HI site and the other ~ 1543 bp downstream of the first ATG codon. A 1.8-kb *Sph*I fragment containing the fission yeast *ura4*$^+$ gene was inserted between the two *Sph*I sites resulting in plasmid pΔINT6, which would remove a 1539 bp segment of *int6*$^+$ open reading frame. This construct, digested by *Xho*I and *Spe*I, was used to disrupt one copy of the *int6*$^+$ gene in a diploid (*h*$^+$/*h*$^-$ *leu1–32/leu1–32 ura4-d18/ura4-d18 ade6-216/210*). Stable Ura$^+$ transformants were selected, and the presence of *ura4*$^+$ at the *int6*$^+$ locus was confirmed by Southern analysis.

Construction of Myc or Green Fluorescent Protein-Tagged int6$^+$

*O*pen *r*eading *f*rame (ORF) of *E*nhanced *G*reen *F*luorescence *P*rotein (EGFP) was amplified by PCR using *pfu* DNA polymerase (Stratagene) and two primer sequences as follows: N-terminus, 5′-d CCGCTCGAGGGGCATGCTAGTAAAGAGAAGAACTTTTC-3′ having a *Xho*I/*Sph*I overhang; C-terminus, 5′-d TCCGAATTCAGCATGCTTTTTGTATAGTTCATCCATGCC-3′ having a *Sph*I/*Eco*RI overhang. The PCR product was digested with *Sph*I and was cloned into *Sph*I linearized pINT6 plasmid, thus generating pINT6-GFP plasmid. The resulting construct was digested by *Xho*I and *Spe*I and was used to knock in Int6-GFP fusion gene in the Δ*int6* haploid in which *int6*$^+$ locus was disrupted with *ura4*$^+$.

The N-terminally Myc-tagged Int6-expressing strain was also generated using a similar strategy except that the *Bam*HI site at + 4 position was utilized to insert the Myc tag at the N-terminus, maintaining the correct reading frame.

Caffeine Sensitivity Assay

The wild-type and Δ*int6* strains were streaked on fresh YEA plates and incubated at 32°C for two days. Cells from YEA plates were inoculated into YEA medium containing 10 mM caffeine and were incubated at 32°C for 12 h. Cells were harvested and washed with 1.2 M sorbitol, followed by staining with DAPI (4′, 6-diamidine-2-phenylindole dihydro-chloride), then examined under a fluorescence microscope enhanced by CCD. For the experiment presented in Figure 10, fresh wild-type cells transformed with empty pREP41, pREP41-INT6, or pREP41-CΔ50 INT6 were streaked on PM plates supplemented with 10 μg/ml thiamine and were grown at 32°C for 12 h. The cells were inoculated into PM medium containing 5 mM caffeine and were grown for another 12 h at 32°C. Cells were harvested, washed with 1.2 M sorbitol, stained with DAPI, and examined under a fluorescence microscope enhanced by CCD.

Cloning and Expression of Full-length Human Int6 Gene in S. pombe

Full-length human INT6 ORF was reverse-transcribed and PCR amplified from total HeLa RNA using Superscript preamplification

system (Life Technologies-BRL Lifetech, GIBCO, Grand Island, NY). Sequences of the two primers used were as follows: N-terminus, 5′-d CCGCTCGAGCATATGGCGGAGTACGACTTGACT -3′ having a *Xho*I/*Nde*I overhang; C-terminus, 5′- dCGCGGATCCGAATTCTCAGTAGAAGCCAGAATCTTGAG -3′ having a *Bam*HI/*Eco*RI overhang. The PCR product was digested with *Nde*I and *Bam*HI, cloned into the same sites of pREP41 to generate the thiamine-repressible pREP41-hINT6 expression plasmid, and sequenced to ensure error-free DNA synthesis. The construct was transformed into Δ*int6* cells, and transformants were selected on PM plates supplemented with 10 μg/ml thiamine.

Assay for Growth Inhibition in Presence of High KCl Concentration

Wild-type and Δ*int6* strains were streaked on fresh YEA plates and incubated at 32°C for two days. Cells recovered from these plates were inoculated into YEA medium containing 1.2 M KCl and were grown in this high-salt medium for ~ 12 h at 32°C. The cells were washed and examined as described under "Caffeine Sensitivity Assay".

β-Glucanase Sensitivity Assay

β-glucanase sensitivity assay was performed following procedures described previously (Levin and Bishop, 1990). Briefly, 5×10^7 cells of WT, Δ*pmk1, or* Δ*int6* cells were harvested from midlogarithmic phase cultures in YEA medium and were washed with and resuspended in a buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, followed by treatment with 100 μg/ml β-glucanase (Zymolyase-20T, ICN) in the same buffer at 32°C with vigorous shaking. Cell lysis was monitored by measuring absorbance at 600 nm.

In Vivo Protein Synthesis

The method used for measurement of in vivo protein synthesis is an adaptation of the method of Sachs and Deardorff (Sachs and Deardorff, 1992). Exponentially growing cultures of wild-type and mutant Δ*int6* strains in YEA medium at 32°C were harvested, resuspended in fresh YEA medium to a cell density of 0.75×10^6 cells/ml, and allowed to grow. When the cells reached the density of $\sim 1 \times 10^6$ cells/ml, ~ 2.5 ml of each culture was treated with 100 μCi of [^{35}S]methionine (1175 Ci/mmol), and cells were allowed to grow with vigorous shaking. At the indicated times, an aliquot (0.8 ml) was withdrawn, mixed with an equal volume of 20% trichloroacetic acid (TCA) containing 1.2 mg/ml unlabeled methionine, and the mixture heated at 95°C for 20 min in glass tubes. After cooling in an ice-water bath, the precipitated proteins were filtered through GF/C filters (Whatman, Maidstone, United Kingdom), which were then washed several times with 10% ice-cold TCA, followed by a final washing with 95% ethanol. The dried filters were then assayed for ^{35}S-radioactivity by counting in Econoflour (Packard Instrument, Meriden, CT) in a liquid scintillation spectrometer.

Polysome Profile Analysis

Exponentially growing cultures of the wild-type strain and the Δ*int6* strains in YEA medium at 32°C were harvested, resuspended in fresh YEA medium to a final cell density of 0.75×10^6 cells/ml, and allowed to grow. When the cells reached the density of $\sim 1 \times 10^6$ cells/ml, a 70-ml aliquot of each culture was treated with cycloheximide (50 μg/ml), rapidly chilled in an ice water bath, and then harvested. The cells were washed twice with LHB buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 30 mM $MgCl_2$, and 50 μg/ml cycloheximide). The washed cells were then suspended in 0.5 ml of LHB buffer, lysed by vortexing with an equal volume of glass beads, then treated with an additional 0.5 ml of LHB buffer. The lysates were clarified by centrifugation at 12,000 $\times$ *g* for 15 min, and equivalent amounts of A_{254} absorbing material (~ 10 A_{254} units) were layered on 11 ml of 7 to 47% (wt/vol) sucrose gradients in TMN buffer (10 mM Tris-Acetate, pH 7.0, 12 mM $MgCl_2$, 50 mM NH_4Cl) and centrifuged at 40,000 rpm for 2.5 h at 4°C in a Beckman SW41 rotor (Beckman, Fullerton, CA). The gradients were fractionated in an ISCO density gradient fractionator, and the absorbance profile at 254 nm was analyzed in an ISCO (Lincoln, NE) UA-5 absorbance monitor.

Association of Fission Yeast Int6 with 40S Ribosomal Particles

A 100-ml culture of fission yeast cells harboring N-terminally Myc tagged Int6 were grown to midlogarithmic phase in YEAU medium. Cells were harvested and lysates prepared as described under "Polysome Profile Analysis" (see above). Approximately 10 A_{254} units of the lysates were layered on 11-ml of 5–30% (wt/vol) sucrose gradients in TMN buffer (10 mM Tris-Acetate, pH 7.0, 12 mM $MgCl_2$, 50 mM NH_4Cl) and were centrifuged at 40,000 rpm for 2.5 h at 4°C in a Beckman SW41 rotor. In a parallel tube, a preformed 43S preinitiation complex (40S·eIF3·AUG·[^{35}S]Met-tRNA$_f$·eIF2·GTP) formed with purified mammalian initiation components was also analyzed to define the position of sedimenting 40S particles. Fractions (500 μl) were collected from the bottom of each tube, and were treated with trichloroacetic acid (16% final concentration). The precipitated proteins in each fraction were resuspended in 50 μl of SDS-gel loading buffer, then boiled, and a 10 μl aliquot was analyzed by SDS-PAGE (10% gel) followed by immunoblotting using appropriate antibodies. In the gradient tube containing the 43S preinitiation complex, an aliquot of each fraction was counted in Aquasol (Packard Instrument, Meriden, CT) in a liquid scintillation spectrometer to determine the position of the 43S preinitiation complex.

Other Methods

eIF3 was purified from rabbit reticulocyte lysates as described by Chaudhuri *et al.* (1997a). The purified eIF3 preparation contained the p48 polypeptide (INT6 protein) as judged by immunoblot analysis using antihuman INT6 antibodies as probe. The 43S preinitiation complex containing bound [^{35}S]Met-tRNA$_f$ was prepared as described by Chaudhuri *et al.* (1997b) as follows. A reaction mixture (225 μl), containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM 2-mercaptoethanol (TKM buffer), 15 μg of bovine serum albumin, 40 pmol of [^{35}S]Met-tRNA$_f$ (17,500 cpm/pmol), 18 μM GTP, and 5 μg of purified rabbit reticulocyte eIF2, was incubated for 4 min at 37°C to form the [^{35}S]Met-tRNA$_f$.eIF2.GTP ternary complex. The reaction mixture was then supplemented with 1.8 A_{254} units of purified 40S ribosomal subunits, 0.6 μg of purified eIF1A, 5 μg of purified eIF3, 0.1 A_{254} unit of AUG, and $MgCl_2$ (1 mM final concentration). After incubation at 37°C for 4 min, the reaction mixture was chilled in an ice-water bath, and the $MgCl_2$ concentration was raised to 5 mM, then analyzed by 5–30% sucrose gradients in TKM buffer containing 5 mM $MgCl_2$ as described (Chaudhuri *et al.*, 1997b). Rabbit polyclonal antihuman INT6 antibodies are a kind gift from Dr. Pierre Jalinot of Laboratoire de Biologie Moléculaire et Cellulaire, Lyons, France. The antibodies were raised against the C-terminal 20 amino acids of the protein (Desbois *et al.*, 1996).

RESULTS

Molecular Cloning of Fission Yeast int6$^+$

Comparison of the predicted amino acid sequence of each of the 10 mammalian eIF3 subunits with the derived protein sequences in *S. pombe* and *S. cerevisiae* genomic databases showed that only five of the mammalian eIF3 subunits have corresponding homologues in the budding yeast, *S. cerevisiae*. In contrast, except p35, all other subunits of mammalian

Table 1. Structural homologues of mammalian eIF3 subunits in yeasts

H. sapiens	*S. pombe*[a]	Identity/similarity[b]	*S. cerevisiae*	Identity/similarity[b]	*S. c.* Core[c]
p170	c17D11/spTif32[d]	31/52	Rpg1p/Tif32p	25/46	yes
p116	c25G10/spPrt1[d]	38/57	Prt1p	31/52	yes
p110	c4A8/spNip1[d]	38/58	Nip1p	29/48	yes
p66	c637/Moe1/sp p66[d]	41/58	ND	N/A	
Int6	c646/spInt6[d]	39/57	ND	N/A	
p47	c4C3/sp p47[d]	35/58	ND	N/A	
p44	c18H10/spTif35[d]	39/59	Tif35p	33/53	yes
p40	c1682/sp p40[d]	23/46	ND	N/A	
p36	c4D7/Sum1/sp Tif34[d]	49/67	Tif34p	44/64	yes
p35	ND	N/A	ND	N/A	

ND, No detectable homologue present; N/A, Not applicable.

[a] Cosmids for the fission yeast homologues/gene names (if known)/proposed names.

[b] Identity/similarity with human eIF3 subunits as common denominator.

[c] Shown are the members of proposed *S. cerevisiae* core eIF3 complex.

[d] Denotes the proposed gene names for fission yeast homologs of eIF3 subunits.

eIF3 including INT6 (p48) have structural homologues in the fission yeast, *S. pombe* (Table 1). The *int6*$^+$ gene was found on a cosmid clone (No. 646) that was previously mapped on chromosome II of *S. pombe* genome (Mizukami *et al.*, 1993). The Int6 protein is highly conserved among species throughout the length of the protein. Int6 from *S. pombe*, *C. elegans*, *Drosophila*, mouse, and human were aligned for maximum homology by Jotun Hein method (Figure 1). *S. pombe* Int6 is 43% identical and 59% similar to its mammalian counterparts. The fission yeast Int6, however, is ~ 15% longer than its higher eukaryotic homologues due to the presence of extra amino acids at the extreme N-terminus as well as near the C-terminus. Following appropriate enzymatic digestion of the cosmid No. 646, the entire *int6*$^+$ gene, flanked by surrounding genomic sequence, was identified in a 3.5-kb *Spe*I-*Xho*I fragment. This fragment was inserted into the appropriate restriction sites of pBluescript SK (-) to generate pINT6. Analysis of the nucleotide sequence of the 3.5-kb *Spe*I-*Xho*I fragment indicated that the gene has a 58-bp long intron 34-bp downstream of the start ATG. The initiation ATG codon at + 1 is preceded by a translational stop codon TGA at position −15 and fairly satisfies the criteria for consensus of translational start sites $^{-3}$GAU*AUG*G^{+4} (the start codon is italicized) (Kozak, 1999).

Deletion of int6$^+$

The *int6*$^+$ gene was replaced with the fission yeast *ura4*$^+$ gene as described under MATERIALS AND METHODS. The replacement (Figure 2A) would allow expression of, at most, the first one and the last two amino acids of the Int6 protein and thus would not cause any phenotypes due to the remaining part of the *int6*$^+$ gene. After transformation of wild-type diploid cells with a plasmid (designated pΔINT6) that carried the *ura4*$^+$ gene in place of the *int6*$^+$ gene, stable Ura$^+$ transformants were isolated. Genomic DNAs of several transformants were subjected to Southern blot analysis to screen for heterozygous diploids with one wild-type *int6*$^+$ gene and one disrupted (*h*$^+$/*h*$^-$ *leu1–32/leu1–32 ura4-d18/ura4-d18 int6*$^+$*::ura4*$^+$/+ *ade6–216/210*). The heterozygous diploid was then sporulated for tetrad analysis. Of the 13 tetrads, eight yielded all four viable spores in which the Ura$^+$ marker segregated into 2:2; indicating *int6*$^+$ gene is not essential for viability. To further confirm the disruption of the *int6*$^+$ locus, Southern and Northern analyses were carried out following standard protocols. The *Sal*I restriction digest followed by hybridization of the probe (shown in Figure 2A) would identify a 1.9-kb DNA fragment in wild-type *int6*$^+$ locus, whereas it would identify a 4.7-kb fragment from the disrupted locus. As anticipated above, a single 1.9-kb fragment was identified in the wild-type diploid (Figure 2B, lane 1), whereas a 4.7-kb fragment in addition to the 1.9-kb fragment was observed in the heterozygous diploid (lane 2). Analysis of four segregants from a tetrad of the heterozygous diploid identified the 4.7-kb DNA fragment in two of the Ura$^+$ segregants (lanes 3 and 5), whereas in the other two segregants that were *ura4*$^-$, the 1.9-kb band was detected (lanes 4 and 6). Northern analysis of the four segregants further confirmed the disruption of the *int6*$^+$ locus. The *int6*$^+$ transcript was detected in the wild-type diploid, the heterozygous diploid as well as in the two *ura4*$^-$ segregants (Figure 2C, lanes 1, 2, 4 and 6). In contrast, no *int6*$^+$ transcript was detected in the two Ura$^+$ segregants (lanes 3 and 5), although approximately similar amounts of total yeast RNA (20 μg) were examined (Figure 2C, lower panel). These results indicated that 1) the *int6*$^+$ gene was successfully replaced by the *ura4*$^+$ gene, and 2) the resulting disrupted strains that expressed no *int6*$^+$ gene were viable. The *int6*$^+$-disrupted haploid, henceforth, will be referred to as Δ*int6*.

Although the *int6*$^+$ gene is not essential for growth and viability, Δ*int6* cells grew more slowly than a wild-type strain. At 32°C, an optimal growth temperature for fission yeast, the generation times of a wild-type and Δ*int6* cells in YEA medium were 2.0 ± 0.2 h and 2.56 ± 0.2 h, respectively (Figure 3A). The growth of Δ*int6* strain was particularly

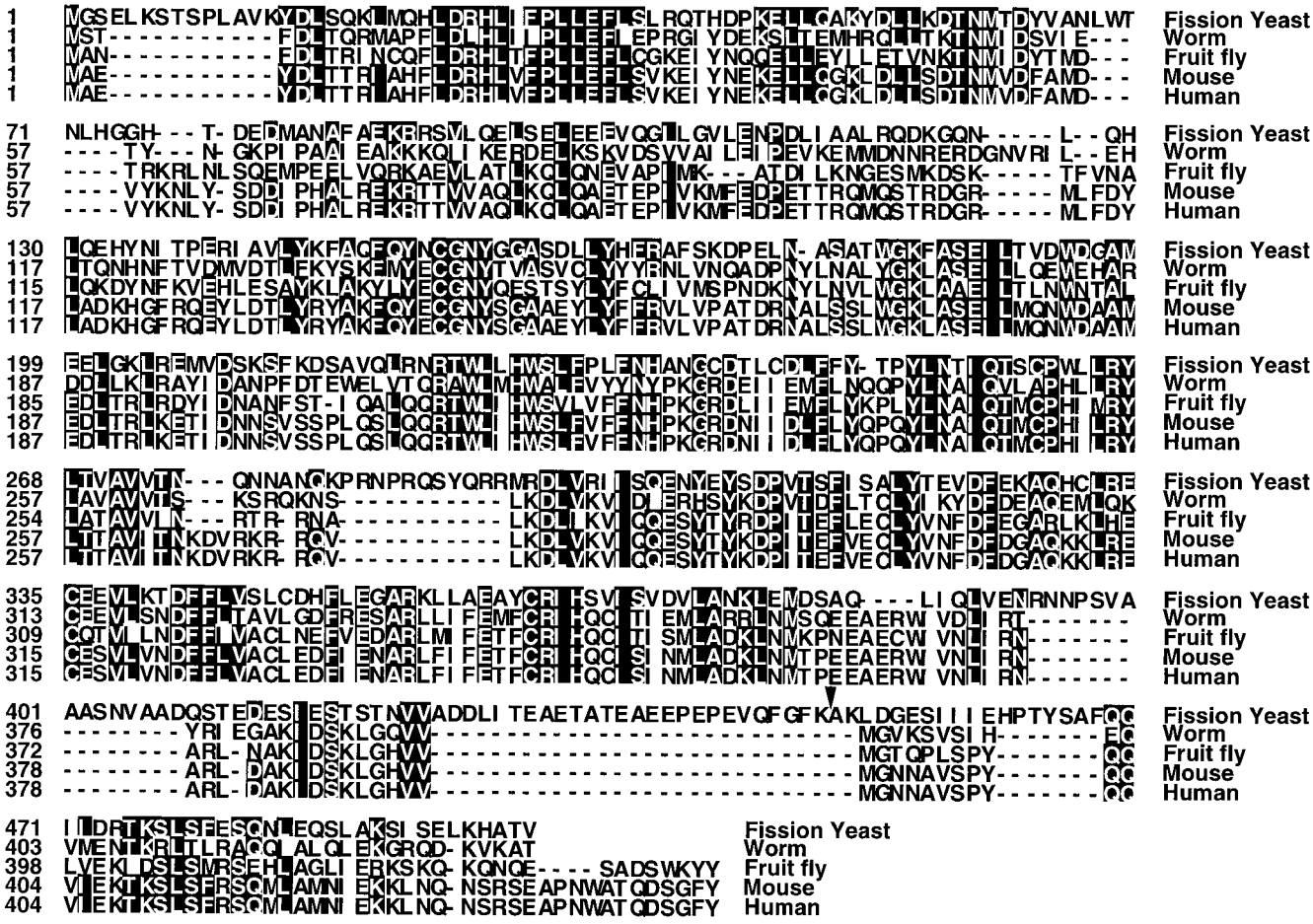

Figure 1. Comparison of Int6 sequences. Alignment of the predicted fission yeast Int6 amino acid sequence with its structural homologues from worm, fruit fly, mouse, and human. The conserved residues are highlighted. The arrowhead marks the position where CΔ50 ends.

impaired in *S. pombe*-liquid minimal medium (PMA). In this medium, while the wild-type strain grew at a rate similar to that in YEA liquid medium, the generation time of Δ*int6* strain in PMA was greater than 3.5 h. The slow growth phenotype of Δ*int6* could be rescued by introduction of the wild-type *int6*$^{+}$ gene or to a lesser extent by the human Int6 gene provided from an extrachromosomally replicating plasmid (Figure 3B).

We also constructed strains that express Int6 tagged with Myc-epitope or GFP. In the parental plasmid, pINT6, appropriate restriction sites were generated into which Myc-epitope or GFP were inserted. The resulting plasmids were used to replace the disrupted *int6*$^{+}$ gene with *int6*$^{+}$ tagged with Myc-epitoope or GFP (Figure 2A). The ura^{-} transformants that had lost the *ura4*$^{+}$ gene due to the replacement were collected and further examined for the structure of the *int6*$^{+}$ gene. Using Southern and Northern blotting as well as PCR, we confirmed that the replacement was successful and that it produced strains expressing tagged Int6 protein, but were otherwise identical to the parental strain (data not shown). These strains did not show any phenotypes conferred by Δ*int6* (see below), indicating that Int6 tagged with Myc-epitope or GFP are biologically active.

Association of Int6 with 40S Ribosomal Subunits

Eukaryotic translation initiation factor 3 (eIF3), purified from mammalian cell lysates, was reported to consist of 10 major polypeptides (Hershey *et al.*, 1996). One of these polypeptides, p48 was shown to be INT6 based on cDNA sequence analysis (Asano *et al.*, 1997). Consistent with this observation, we also found that purified mammalian eIF3 ((Benne and Hershey, 1976; Chaudhuri *et al.*, 1999) contained INT6 polypeptide (Figure 4A, inset). However, it should be noted that the level of INT6 in this preparation was not stoichiometric to the other subunits of eIF3 (see Discussion). Characterization of mammalian eIF3 has shown that in crude mammalian cell-free extracts, eIF3 is found exclusively bound to 40S particles (Smith and Henshaw, 1975; Thompson *et al.*, 1977). In vitro studies using purified initiation factors have also shown that eIF3 binds to 40S subunits in the absence of all other initiation components to form the 40S.eIF3 complex that is stable to sucrose gradient centrifugation (Benne and Hershey, 1976; Chaudhuri *et al.*,

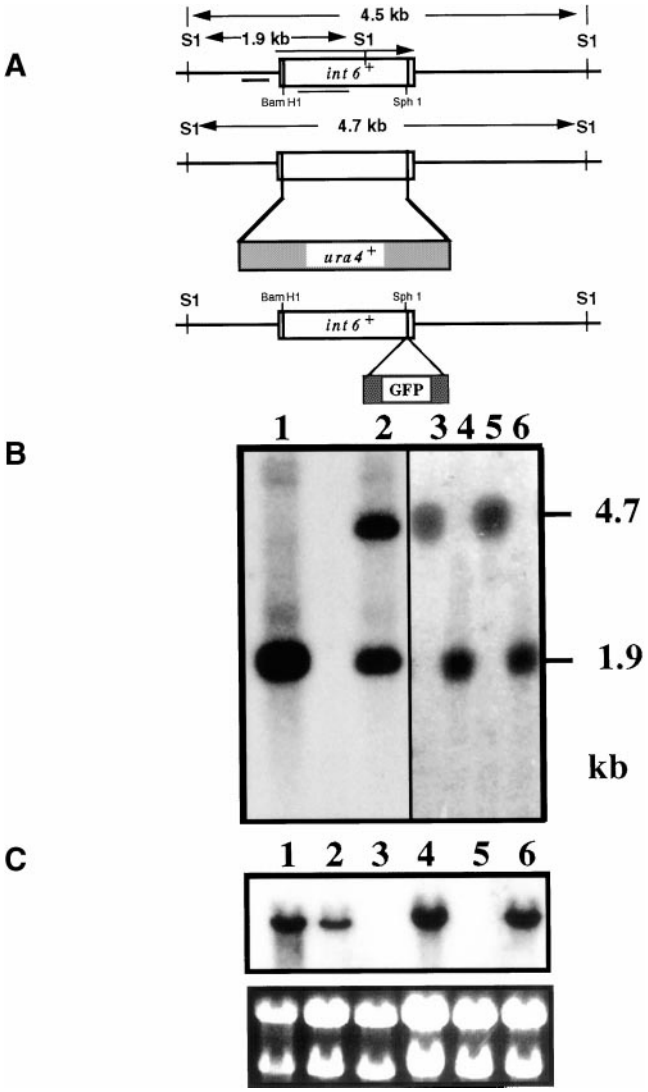

Figure 2. Analysis of *int6*$^+$ disrupted strain. (A) Strategy of disruption of the *int6*$^+$ gene and construction of C-terminally GFP tagged or N-terminally Myc tagged Int6. The entire *int6*$^+$ gene was replaced with the fission yeast *ura4*$^+$ gene in a diploid. The *Sal*I restriction sites are shown as S1. The GFP or Myc tag is inserted in the *Sph*I or *Bam*HI site, respectively, as described in MATERIALS AND METHODS and inserted in place of the *ura4*$^+$ gene. (B) Confirmation of disruption by Southern blotting. The replacement was confirmed by Southern blotting using a ^{32}P-labeled PCR product from the 5′UTR of *int6*$^+$ locus as a probe (thick bar). Genomic DNAs of a wild-type diploid (lane 1), heterozygous diploid (*int6*$^+$:: *ura4*$^+$/+) (lane 2), a set of four segregants from the heterozygous diploid (lanes 3 to 6) were examined. (C) Confirmation of disruption by Northern blotting. Northern blot was performed with 20 μg of total RNA from a wild-type diploid (lane 1), the heterozygous diploid (int6$^+$ :: *ura4*/+) (lane 2), and a set of four segregants from the heterozygous diploid (lanes 3 to 6) using a probe generated from within the *int6*$^+$ ORF (thin bar). The lower panel shows the ethidium bromide stained RNA gel.

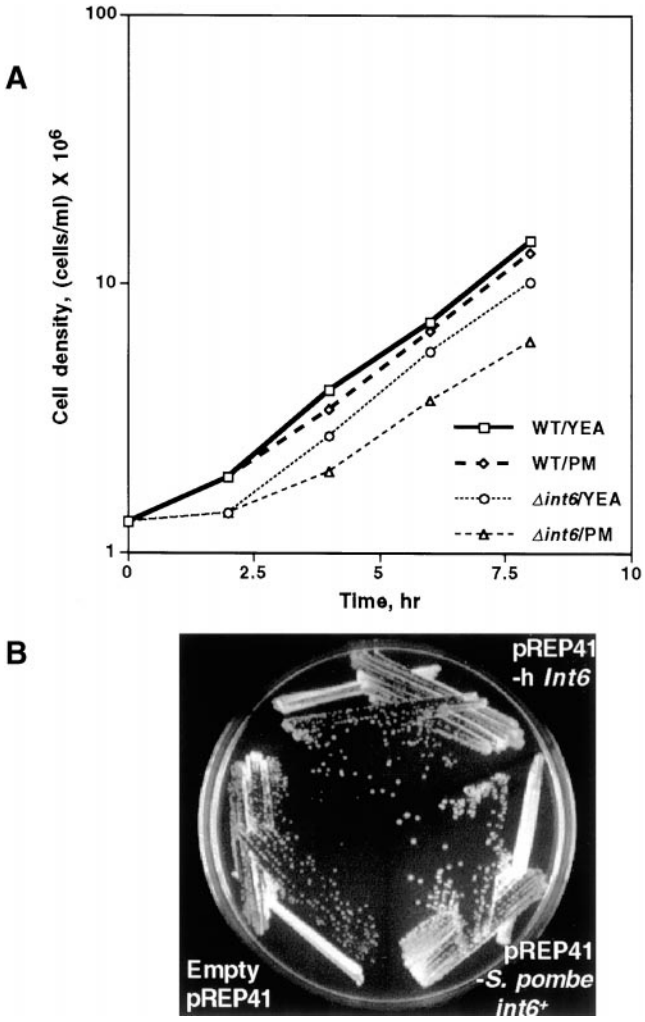

Figure 3. Slow growth of Δ*int6* strain and rescue by the wild-type fission yeast or human *int6*$^+$ gene. (A) Growth curve. Cells were grown to $\sim 5 \times 10^6$ cells/ml at 32°C and then diluted to 1.3×10^6 cells/ml at time 0. The cell density was monitored over a 6 h period. (B) Δ*int6* strain was transformed with either empty pREP41 vector or vectors bearing full length fission yeast *int6*$^+$ or human Int6 gene. Transformed cells were streaked on a PM plate.

1999). The initiator Met-tRNA$_f$ (as Met-tRNA$_f$.eIF2.GTP ternary complex) then binds to the 40S.eIF3 particle to form the 40S preinitiation complex (40S.eIF3. Met-tRNA$_f$.eIF2.GTP), which subsequently recognizes the AUG codon of the mRNA to form the 40S initiation complex (40S.eIF3.mRNA.Met-tRNA$_f$.eIF2.GTP). These properties of mammalian eIF3 prompted us to examine the association of Int6 with 40S particles in fission yeast cells. We reasoned that if fission yeast Int6, like its mammalian counterpart, is a subunit of eIF3, it should remain bound to the 40S particles under conditions where mammalian eIF3 containing INT6 as one of its subunits remains bound to 40S particles.

Cell-free extracts of a strain of *S. pombe* expressing N-terminally Myc-tagged Int6 were subjected to sucrose gradient centrifugation, and the gradient fractions were assayed for Int6 by Western blot analysis. A 43S preinitiation complex (40S.eIF3. Met-tRNA$_f$.eIF2.GTP), formed by incubating 40S ribosomal subunits with purified mammalian eIF3 and

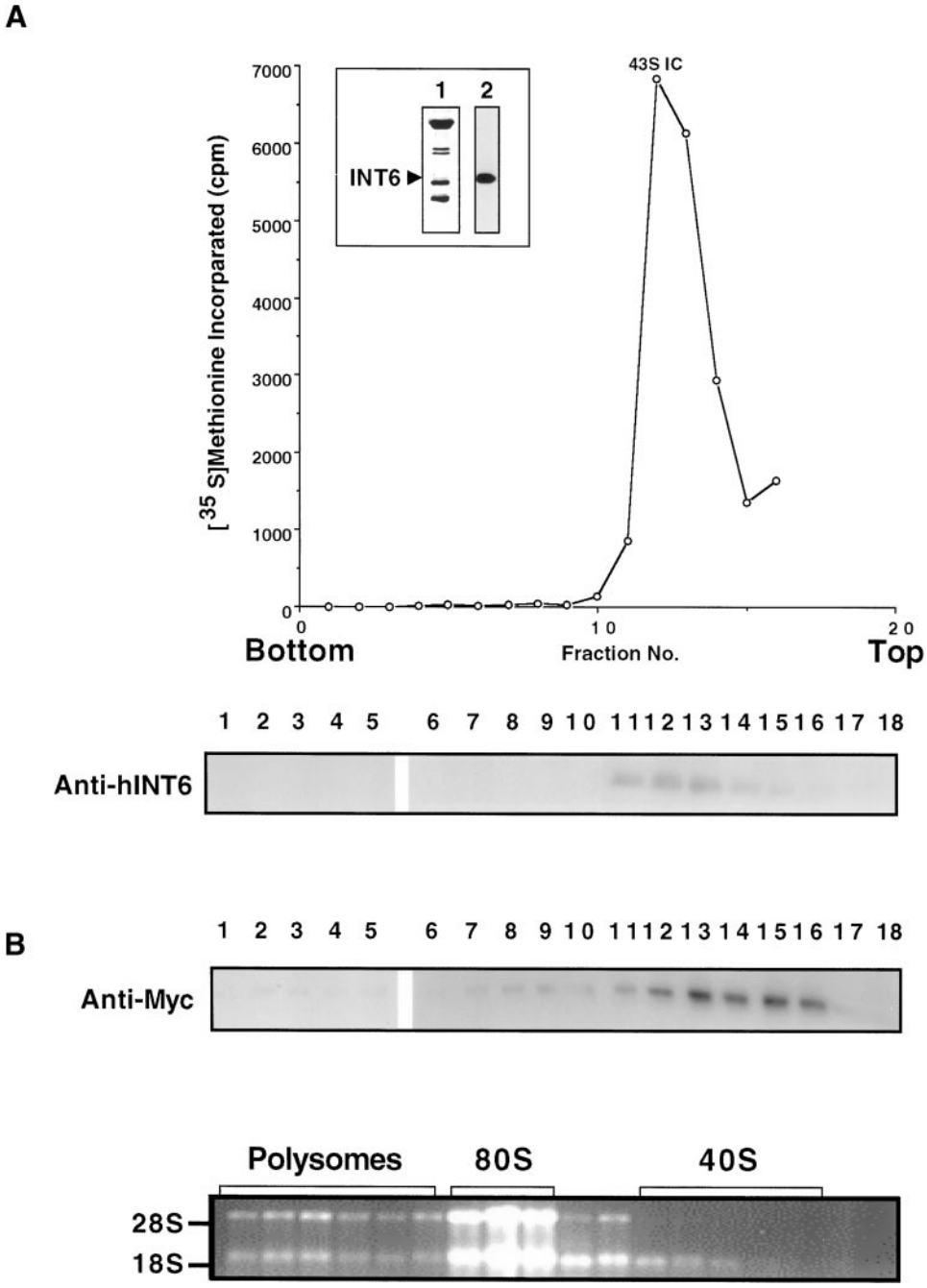

Figure 4. Association of Int6 with 40S ribosomal particles in yeast cells. (A) Mammalian INT6 sediments with the 43S preinitiation complex. A 43S preinitiation complex containing bound [^{35}S]Met-tRNA$_f$ and formed in presence of purified mammalian eIF3, as described in MATERIALS AND METHODS, was analyzed by 5–30% (wt/vol) sucrose gradients. Aliquots of the gradient fractions were assayed for ^{35}S-radioactivity by scintillation counting to determine the sedimentation position of the 43S preinitiation complex (43S IC). Each gradient fraction was also analyzed by Western blotting using antihuman INT6 antibodies as probes. *Inset*. Approximately 300 ng of purified rabbit reticulocyte eIF3 (Chaudhuri *et al.*, 1997) were subjected to SDS-15% PAGE. The gel was treated with silver stain reagents (lane 1) or analyzed by Western blotting using a 1:2000 dilution of polyclonal antihuman INT6 rabbit antibodies (lane 2). The arrowhead indicates the position of INT6 polypeptide, which migrates at the expected position. A set of molecular weight standards was run in parallel lanes (not shown). (B) Fission yeast Int6 cosediments with 40S particles. An exponentially growing culture of fission yeast cells expressing N-terminally Myc tagged Int6 was harvested, the cells were washed and lysed, and the cell lysates (10 A_{254} units) were subjected to 5–30% sucrose gradient centrifugation to separate polysomes, free 80S, 60S, and 40S ribosomes, as described in MATERIALS AND METHODS. Fractions (0.5 ml each) from the gradient were collected and proteins from 0.25 ml of each fraction were precipitated with 16% trichloroacetic acid, analyzed by SDS-PAGE, and immunoblotted with anti-Myc antibodies as probes. Total RNA was also isolated from each gradient fraction by 1% SDS-treatment at 65°C, followed by phenol/chloroform extraction and ethanol precipitation. RNA was analyzed by 1% agarose gel electrophoresis to determine the presence of 28S and 18S rRNAs.

[^{35}S]Met-tRNA$_f$.eIF2.GTP ternary complex, was also analyzed in a parallel gradient tube (Figure 4A). Under these conditions, [^{35}S]Met-tRNA$_f$ bound to 40S ribosomes sedimented with the 40S particles and thus defined the position of 40S ribosomal subunits. Western blot analysis of each gradient fraction showed that Myc-tagged fission yeast Int6 and mammalian Int6 sedimented at a position where the 43S preinitiation complex also sedimented (Figure 4B, upper panel). It should be noted that, in agreement with the results published previously (Chaudhuri *et al.*, 1999), when the gradient fractions from the 43S preinitiation complex were analyzed by Western blotting using total anti-mammalian eIF3 antibodies (Chaudhuri *et al.*, 1997a) as probes, all the mammalian eIF3 subunits cosedimented with the 43S preinitiation complex (data not shown). Western blot analysis of gradient fractions using antimammalian INT6 antibodies showed that INT6 also sedimented with the 43S preinitiation complex (Figure 4A, lower panel). Further confirmation that fission yeast Int6 sedimented with the 40S particles came from the observation that the Int6 containing fractions con-

tained 18S rRNA, a known constituent of 40S ribosomal subunits (Figure 4B, lower panel). It should be noted that a portion of fission yeast Int6 sedimented in fractions lighter than 40S particles. The possibility exists that N-terminally tagged Int6 has a weaker association with the other subunits of eIF3. Alternatively, association of fission yeast Int6 with the other eIF3 subunits is inherently weaker than its mammalian counterpart.

Int6 Is Not Essential for Translation Initiation

The presence of INT6 in highly purified eIF3 preparations prompted us to examine the effect of deletion of the *int6*$^+$ gene on translation of mRNA in *S. pombe* cells. For this purpose, exponentially growing cultures of Δ*int6* and a wild-type strain were pulsed with [^{35}S]methionine, and the rate of protein synthesis was measured over a 30-min time period. The rate of protein synthesis, measured by incorporation of [^{35}S]methionine into polypeptide chains in Δ*int6* cells, was ~ 30–40% slower than the rate of wild-type cells (Figure 5, panel A). This slower rate of methionine incorporation into cellular proteins in Δ*int6* cells is in keeping with the slower growth rate of Δ*int6* cells as compared with the wild-type strain.

To investigate whether the 30–40% decrease in the incorporation of [^{35}S]methionine was due to slight defects in the initiation phase of protein synthesis, we examined the polyribosome profile of exponentially growing cultures of Δ*int6* and control wild-type cells. It is now well established that, when initiation of translation is slowed or blocked, ribosomes already bound to mRNAs complete translation but, after their release from mRNAs, do not reinitiate the translation process. This leads to a diminution of the size of polysomes and accumulation of 80S ribosomes. When extracts prepared from exponentially growing cultures of Δ*int6* and wild-type control cells were analyzed by sucrose gradient centrifugation, the polyribosome content of both strains showed similar profiles (Figure 5, panel B). However, a slight decrease in the polysome content and a slight increase in free 80S ribosomes were observed for Δ*int6* cells. These results may indicate a small effect of Int6 protein in initiation of translation but rule out that Int6 is essential for the initiation process in global protein synthesis.

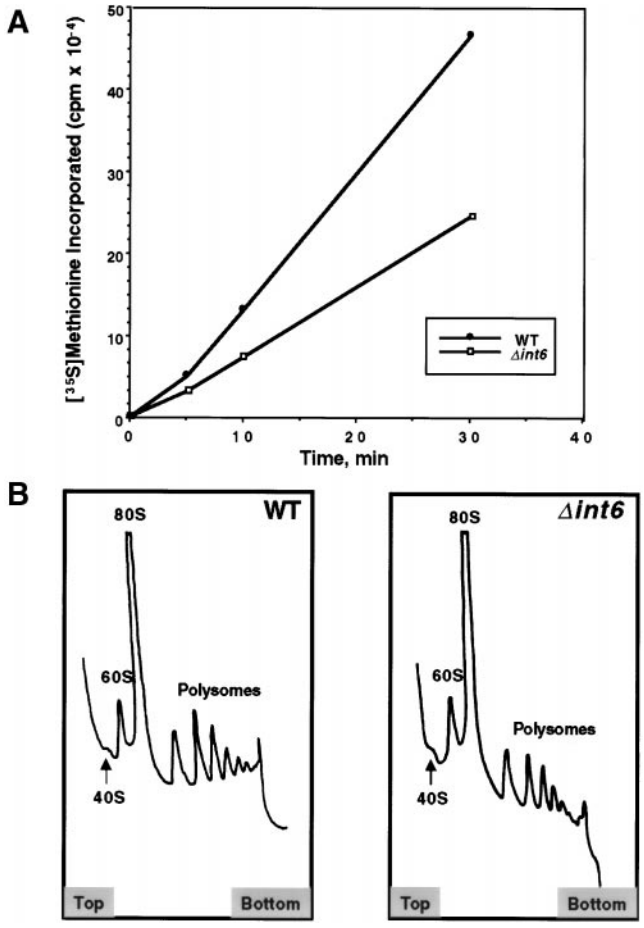

Figure 5. (A) In vivo incorporation of [^{35}S]methionine in wild-type and Δ*int6* fission yeast cells. Total incorporation of [^{35}S]methionine into proteins in a wild-type or Δ*int6* strain was determined. (B) Analysis of polysomes. Cell lysates were prepared from exponentially growing cultures of a wild-type strain (left) or a Δ*int6* strain (right) in YEA medium at 32°C and subjected to 7–47% (wt/vol) sucrose gradient as described in MATERIALS AND METHODS. Each gradient was fractionated in an ISCO gradient fractionator, and the absorbance profile at 254 nm was analyzed in an ISCO UA-5 absorbance monitor.

Int6 Is a Cytoplasmic Protein

Using a strain expressing Int6 tagged with GFP, we determined the cellular localization of the protein. The majority of Int6-GFP was found in cytoplasm. This localization and the intensity of the signal seemed to be homogenous when an asynchronous culture was examined (Figure 6), suggesting that Int6 does not change its level and localization in a manner depending on progression of the cell cycle.

Δint6 Is Hypersensitive to Caffeine

We tested the Δ*int6* strain for sensitivity to a variety of stress conditions such as treatment with UV light, caffeine, thiabendazole, and incubation at 36°C or 20°C. Among these conditions it was observed that Δ*int6* is hypersensitive to caffeine. In the YEA medium containing 10 mM caffeine, a wild-type fission yeast strain grew slowly (Figure 7B). It was also apparent that most of the cells have multiple septa (Figure 8C). The phenotype induced by the drug would suggest that the drug might have an inhibitory effect on maintenance of integrity of cell wall/membrane, or on cytokinesis. The caffeine-induced phenotype was much more pronounced in the Δ*int6* strain. The Δ*int6* cells were unable to form colonies on YEA plates containing 10 mM caffeine (Figure 7B). Microscopic observation revealed that these cells were elongated and lysed (Figures 7A, 8D and 8F). Because Δ*int6* cells lysed in the caffeine containing medium, we speculated that it could become resistant to the drug if the medium contained an osmotic stabilizer such as sorbitol. Indeed, if sorbitol was added to the medium, Δ*int6* strain could form colonies in the presence of 10 mM caffeine at a rate similar to that of the wild-type strain (Figure 7B). Although the drug could have multiple cellular targets and cause various phenotypes, the suppression by sorbitol

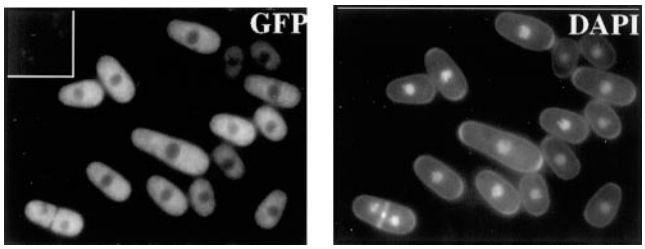

Figure 6. Fission yeast Int6 is localized in the cytoplasm. Fission yeast cells expressing C-terminally GFP-tagged Int6 were grown on YEAU plates along with wild-type cells. Cells were stained with DAPI and were observed under CCD camera either in GFP channel or in DAPI channel. The inset shows the background fluorescence in GFP channel with the wild-type control.

would indicate that the hypersensitivity to the drug is most likely due to a defect in cell wall/membrane caused by lack of Int6 protein.

A similar phenotype, i.e., cell elongation and lysis with multiple septation in the presence of caffeine, has previously been reported for MAPK cascade mutants and the function of the MAPK cascade in maintenance of cell wall integrity has been proposed (Loewith *et al.*, 2000). It has also been shown that these deletion strains are sensitive to high concentration (1.2 M) of KCl in the medium. We observed that Δ*int6* cells were likewise sensitive to 1.2 M KCl concentration in the medium. Whereas a wild-type strain could form colonies on YEA plates containing 1.2 M KCl, the Δ*int6* strain failed to do so (our unpublished results).

It has been reported that the mutants of the MAP kinase cascade form a defective cell wall. Treatment with a cell wall-digesting enzyme, β-glucanase, results in cell lysis more dramatically in the mutants than wild-type strains (Toda *et al.*, 1996; Sengar *et al.*, 1997)]. The observed similarity between Δ*int6* and the mutants of the MAP kinase cascade prompted us to test if Δ*int6* exhibits a similar defect in cell wall integrity. As shown in Figure 9A, deletion of the MAP kinase gene (Δ*pmk1*) exhibited a high sensitivity to β-glucanase, whereas Δ*int6* as well as a wild-type strain were resistant to the enzyme treatment. These results may suggest that the underlying mechanism of caffeine sensitivity conferred by *int6*$^+$ deletion may not be the same as for the MAP kinase cascade mutants.

Δ*int6* Cells Fail to Recover from Saturated Growth

We also observed that the Δ*int6* cells were unable to recover from growth saturation. When they were transferred from an actively growing state to fresh YEA medium, they formed colonies at a slightly reduced rate (Figure 9B) compared with wild-type cells. In contrast, when the Δ*int6* cells were grown to a saturated state and transferred to fresh YEA medium, a major fraction of the cells were unable to form colonies (Figure 9B). Microscopic observation revealed that the Δ*int6* cells transferred from a saturated state were rounded, a phenotype similar to that of starved cells (data not shown). Due to presumed defects in maintenance of cell wall/membrane integrity, the Δ*int6* cells may be defective in their ability to uptake nutrients necessary for recovery from a saturated state.

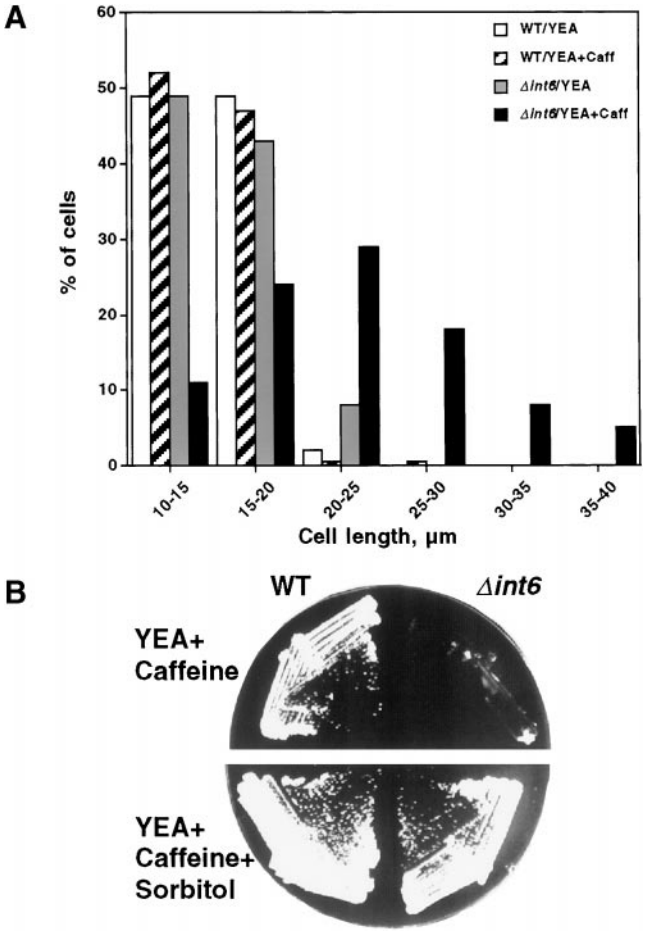

Figure 7. Effect of caffeine on Δ*int6* cells. (A) A histogram of distribution of cell sizes for WT and Δ*int6* cells in YEA media lacking or containing 10 mM caffeine grown at 32°C. (B) Growth of WT or Δ*int6* cells on YEA plates containing 10 mM caffeine in presence or absence of 1.2 M sorbitol.

Negative Effect of a Truncated Int6

In most cases, tumor-causing integration of MMTV genome in the mouse Int6 locus produces different forms of C-terminally truncated INT6 protein. It was therefore hypothesized that overexpression of these C-terminally truncated forms of INT6 driven by viral LTR causes tumors in mice (Marchetti *et al.*, 1995). We examined whether overexpression of a C-terminally truncated Int6 in fission yeast causes any aberrant phenotype. For this purpose, we transformed a wild-type strain with pREP41-CΔ50 INT6 plasmid that would allow overexpression of a truncated Int6 protein lacking the C-terminal 50 amino acids (designated CΔ50). When the transformants were grown in the presence of 5 mM caffeine in PM medium, the cells showed a highly elongated shape (Figure 10). Transformants with a control vector or a plasmid that overexpresses the full-length *int6*$^+$ gene did not show any apparent phenotypes under the same conditions (Figure 10). It should be noted that overexpression of CΔ50 causes a phenotype which is slightly different from that of Δ*int6*. First, the cells expressing CΔ50 exhibited cell

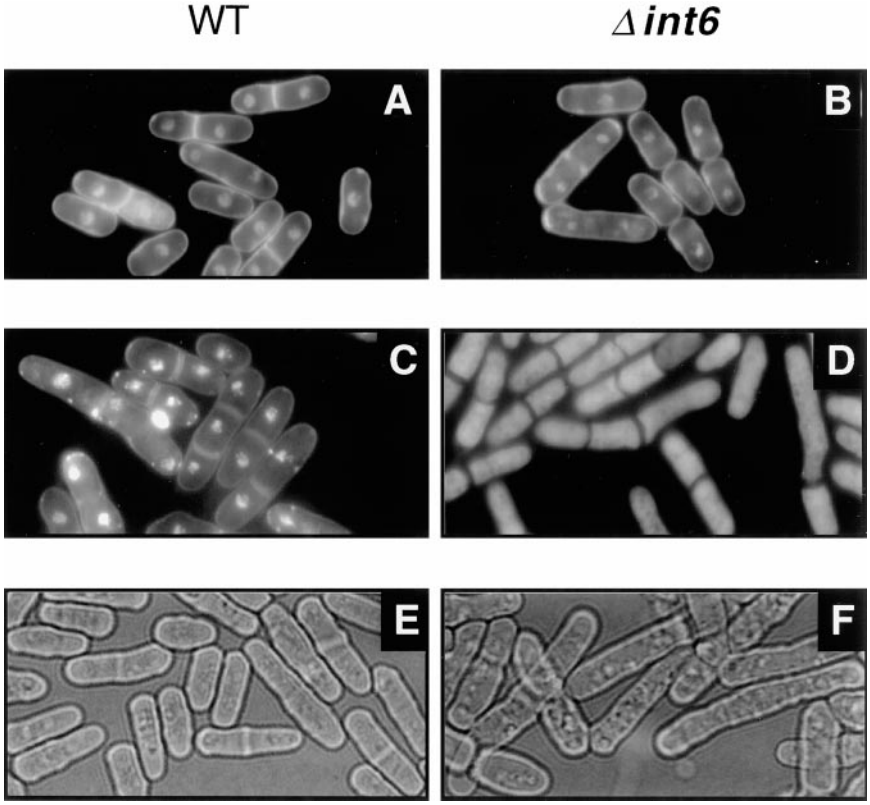

Figure 8. Δint6 cells are sensitive to caffeine. The wild-type strain (A, C, and E) or Δ*int6* (B, D, and F) were grown under the following conditions; YEA medium (A and B), YEA + 10 mM caffeine (C to F). A to D were examined under a fluorescence microscope, while E and F were examined by conventional light microscopy.

elongation at a lower concentration of the drug. Second, the elongated cells in the medium containing caffeine did not lyse. Overexpression of CΔ50 appeared to affect the function of Int6 partially (see DISCUSSION). When CΔ50 was expressed from a single copy gene integrated in the genome, it did not cause the phenotype (our unpublished results).

Defect in Meiosis

In the course of strain construction, we noticed that cross between Δ*int6* and Δ*int6* produced incomplete tetrads frequently. As shown in Figure 11, 30–40% of asci of the Δ*int6* homozygous cross contained less than four spores. The defect was suppressed to a large extent when Δ*int6* was crossed to the wild-type strain.

DICUSSION

Fission Yeast int6$^+$ Gene

In this study we have characterized a fission yeast gene (*int6*$^+$) that is 43% identical to the mammalian INT6 gene. The complementation of deletion of *int6*$^+$ (Δ*int6*) by the human Int6 ORF strongly suggests that they are functionally homologous.

Our interest in the Int6 protein stems from our effort to understand the process of eukaryotic translation initiation and the central role the multi-subunit initiation factor eIF3 plays in this process. Asano *et al.* (1997) reported INT6 as one of the 9–10 polypeptide subunits comprising purified mammalian eIF3. Although in recent years, the molecular genetic techniques in the budding yeast *S. cerevisiae* have been very useful to understand the function of translation initiation factors in vivo, the genome of budding yeast does not encode any genes related to INT6 (Table 1). The fission yeast model system thus offers a unique opportunity to study the functions of Int6 protein by genetic approaches. Assuming that Int6 plays a role with eIF3, fission yeast appears to share a common mechanism for translation initiation and its regulation in mammalian cells.

Association of Int6 with 40S Subunits

The question naturally arises is whether Int6 is a subunit of fission yeast eIF3. To answer this question, it is necessary to isolate and characterize eIF3 from fission yeast. This has not yet been achieved. However, an important property of mammalian eIF3 is its association with 40S subunits in cell-free extracts (Smith and Henshaw, 1975; Thompson *et al.*, 1977). Therefore, if Int6 associates with eIF3, it would remain bound to 40S particles. This work as well as work from Norbury's laboratory has demonstrated that in cell-free extracts of fission yeast, Int6 cosediments with the 40S particles. More directly, fission yeast Int6 can be coimmunoprecipitated with a major component of eIF3, Sum1 (Dr. Chris Norbury, Imperial Cancer Research Fund, University of Oxford, Oxford, United Kingdom; personal communication). These observations suggest that the fission yeast Int6 is either associated with the core subunits of fission yeast eIF3 by protein-protein interaction or the polypeptide is a bona-

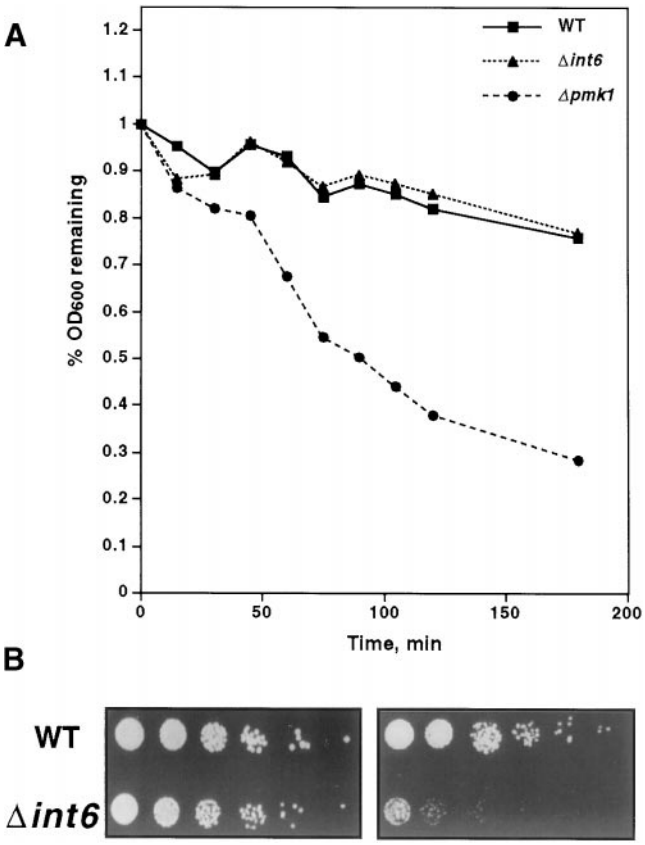

Figure 9. (A) Δ*int6* cells are not hypersensitive to β-glucanase treatment. (B) Failure of Δ*int6* cells to recover from a saturated culture. A wild-type strain (upper panel) or Δ*int6* (lower panel) cells were grown to either logarithmic phase (left) or saturated phase (right) in YEA medium at 32°C. Cells were serially diluted 5-fold each time, spotted on YEA plates, and incubated for 3 days at 32°C.

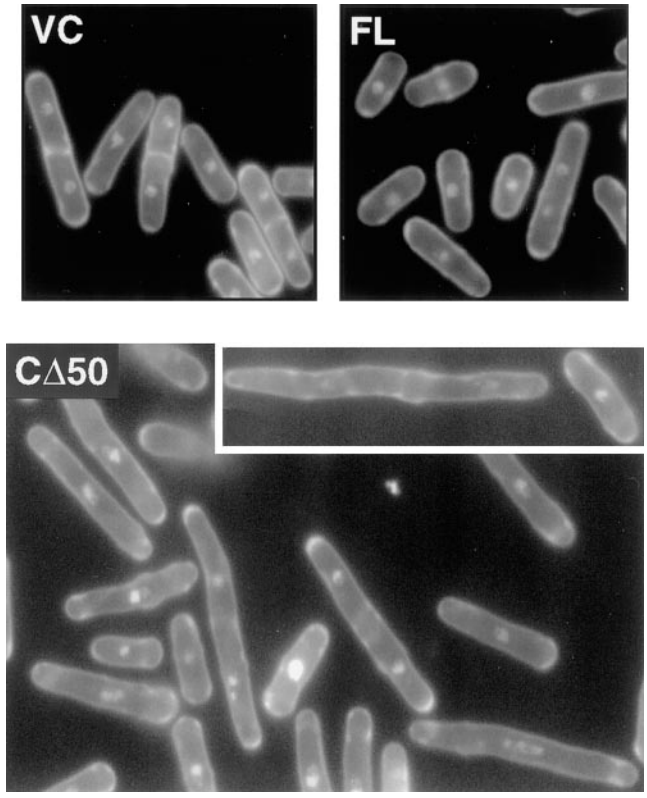

Figure 10. Negative effect of CΔ50-Int6. Wild-type cells were transformed with either empty pREP41 vector (VC) or pREP41 containing either full-length *int6*$^+$ gene (FL) or CΔ50 mutant *int6*$^+$ (CΔ50) gene. Transformants were grown in PM medium for 12 h and were inoculated in PM medium containing 5 mM caffeine. Inset: Occasionally, some cells harboring pREP41-CΔ50 INT6 grew unusually long in caffeine containing medium.

fide subunit of eIF3. It should however, be noted that when we purified eIF3 from rabbit reticulocyte lysates, INT6 was found not to be stoichiometric with the other subunits of eIF3 (Figure 4A). Therefore, the possibility also exists that INT6 associates only with a subset of rabbit eIF3.

Role of int6$^+$ *in Translation Initiation*

We show that fission yeast cells lacking Int6 (Δ*int6*) support protein synthesis well exhibiting polysome-ribosome profiles similar to wild-type cells. If Int6 played an essential role in initiation of global protein synthesis, its absence in yeast cells would have caused extensive breakdown of polysomes with simultaneous increase in free 80S ribosomes as was observed in *S. cerevisiae* cells depleted of any essential translation initiation factor (Maiti and Maitra, 1997). These observations argue against the idea that Int6 is essential for initiation of global protein synthesis. It should, however, be emphasized that Δ*int6* strain showed ~ 30–40% slower rate of [^{35}S]methionine incorporation as compared with a wild-type strain. In view of our observation that the *int6*$^+$ gene is required for maintenance of the integrity of cell wall/membrane, the possibility exists that the lower rate of [^{35}S]methionine incorporation into Δ*int6* yeast cells is a reflection of poorer uptake of amino acids into Δ*int6* yeast cells as compared with *int6*$^+$ cells. Alternatively, it remains possible that Int6 is required either for translation of a small subset of mRNAs or for optimal efficiency of the rate of initiation of translation of all mRNAs. Analysis of phenotypes conferred by Δ*int6* has indicated that Δ*int6* exhibits a defect in the integrity of cell wall/membrane. The Δ*int6* phenotype would support a model in which fission yeast Int6 regulates translation initiation of a subset of proteins that are required for maintenance of the integrity of cell wall/membrane.

Caffeine Hypersensitivity and Other Phenotypes of Δ*int6*

One of the apparent phenotypes conferred by Δ*int6* is the hypersensitivity to caffeine. Although the drug probably targets multiple cellular components and causes various phenotypes, it has been reported that the primary effect of caffeine on the fission yeast cell cycle is an inhibition of cytokinesis (Kumada *et al.*, 1996). Consistent with this previous study, we have observed an inhibitory effect of the

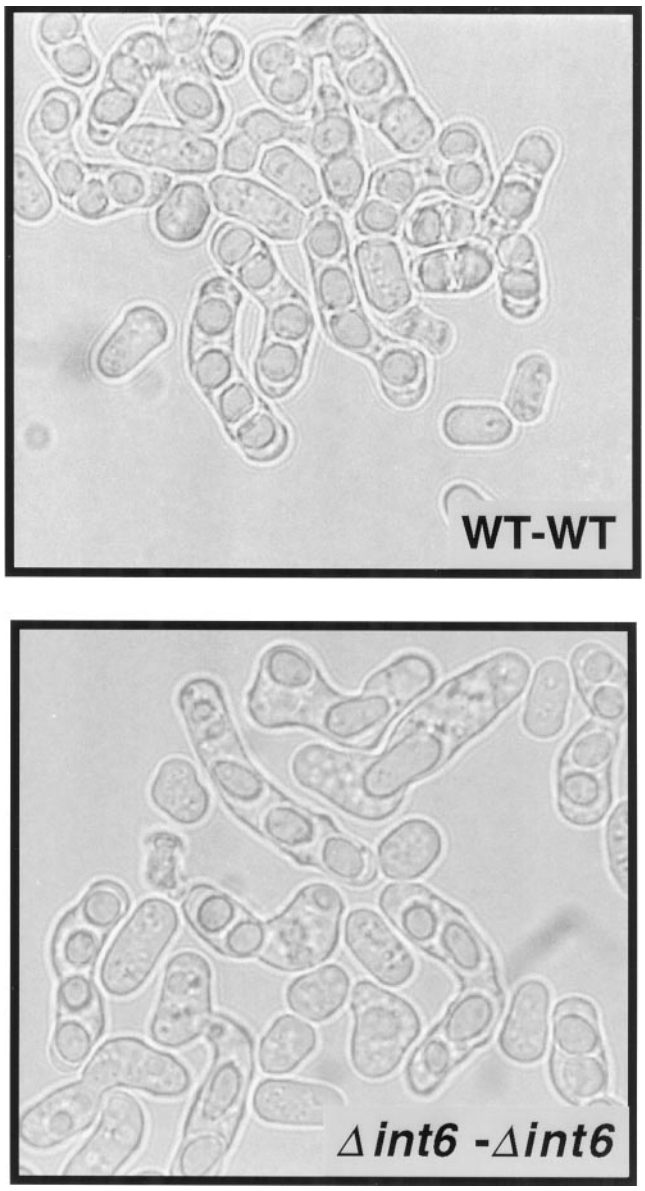

Figure 11. Defective meiosis in Δ*int6*. Crosses between wild-type strains **(**WT-WT) or Δ*int6* strains (Δ*int6* -Δ*int6*) were observed under a conventional light microscope.

drug on cytokinesis in the wild-type strain. The caffeine-induced phenotype is much more prominent in the Δ*int6* strain. The hypersensitivity is suppressed by addition of sorbitol to the media. The suppression strongly implies that Δ*int6* loses its viability due to osmotic stress caused by the drug. Other negative effects that could be caused by the drug would not directly result in the hypersensitivity. Δ*int6* also confers a slow growth rate, sensitivity to KCl, inability to recover from a saturated state and a defect in meiosis. We speculate that Δ*int6* may form a defective cell wall/membrane which causes the phenotypes in the multiple biological processes.

It has recently been reported that the fission yeast MAP kinase cascade plays a role in the maintenance of cell wall/membrane (Loewith *et al.*, 2000; Sugiura *et al.*, 1999; Toda *et al.*, 1996). Three kinases, namely Mkh1, Pek1/Skh1, and Pmk1/Spm1, form a sequential signaling cascade (Loewith *et al.*, 2000; Sugiura *et al.*, 1999; Toda *et al.*, 1996). Deletion of any one of these genes causes phenotypes similar to those of Δ*int6*. First, deletion causes cell elongation in presence of caffeine (Loewith *et al.*, 2000). Second, it causes inhibition of growth in media containing KCl (Loewith *et al.*, 2000). Finally, deletion of $pmk1^+$ causes a defect in recovery from a saturated state (Toda *et al.*, 1996). On the other hand, while the mutants in the MAP kinase cascade form cell wall that is sensitive to treatment with β-glucanase, cell wall of Δ*int6* is resistant to this treatment. Likewise, the mutants in the MAP kinase cascade are not defective in meiosis. These results would suggest a partial overlap in the function of Int6 and the MAP kinase cascade. Further study would shed light on the functional link between the MAP kinase cascade and the Int6 protein.

Effect of a Truncated int6 Protein

Integration of MMTV in the mouse Int6 locus results in expression of a truncated form of the INT6 protein (Marchetti *et al.*, 1995). At present, it is not clear how a truncated INT6 protein induces tumor formation in the mouse mammary epithelial tissue. MMTV may simply disrupt the biological activity of the affected Int6 gene causing a reduction in the dose of the normal gene product. Alternatively, a truncated form of INT6 may have a dominant negative or active effect leading to hyperplasia in the mouse mammary gland. In this study, we have tested if a truncated $int6^+$ gene has a negative effect in fission yeast. For this purpose, we have expressed several versions of truncated Int6 proteins in the wild-type strain. One such construct, which overexpresses a truncated Int6 protein lacking the C-terminal 50 amino acids (designated CΔ50), causes a phenotype. In presence of caffeine, wild-type cells expressing CΔ50-Int6 protein exhibit a highly elongated cell shape. Interestingly, overexpression of CΔ50 can cause the phenotype in a medium containing 5 mM caffeine, whereas Δ*int6* strain expressed the phenotype at a higher concentration of the drug,10 mM. In addition, CΔ50 does not cause other phenotypes observed in Δ*int6*. When CΔ50 is overexpressed, it may replace the wild-type Int6 and abrogate the function.

Based on the assumption that Int6 may play a role with eIF3 in regulation of translation initiation of a subset of proteins, we would interpret the discrepancy between Δ*int6* and CΔ50 as follows: While eIF3 without Int6 fails to initiate translation of all proteins in the subset, eIF3 with CΔ50 can regulate translation initiation of a limited number of proteins in the subset. The difference in protein composition may lead to the discrepancy in expression of the phenotype. It should also be noted that overexpression of the human Int6 can suppress the slow growth of Δ*int6*, but not the hypersensitivity to caffeine. Thus, the human Int6 in fission yeast is partially functional. A lower level of expression of CΔ50 did not cause any phenotype, suggesting it is not dominant negative.

Implication in Tumor Biology

The Int6 gene is a common integration site for the mouse mammary tumor virus (MMTV). A human homologue of Int6 is located on chromosome region 8q22-q23. Examination of this allele revealed loss of heterozygosity (LOH) in 11 of 39 (28%) of the tumor samples. Because single-strand conformation and hybrid mismatch analysis of the remaining allele in these tumor DNAs failed to detect any mutations, it has been concluded that the target gene for LOH must be closely linked to Int6 (Miyazaki *et al.*, 1997). Thus, it is likely that mutations in the human Int6 also cause tumors.

In this study we have shown that the fission yeast *int6*$^+$ is required for maintenance of the integrity of cell wall/membrane. Assuming that the human INT6 functions in a similar biological process, we hypothesize that mutations in the mammalian Int6 gene cause an abnormal membrane structure, which might result in defective endocytosis. Ligand binding followed by its internalization is often used as a signal, which regulates cell growth. The Int6-induced tumor cells may not be able to correctly process such a signal, which negatively regulates cell division. In higher eukaryotes, a number of genes required for endocytosis has been characterized as tumor suppressor genes or oncogenes (Floyd and De Camilli, 1998). It should be noted that the fission yeast Δ*int6* cells fail to recover from a saturated state and exhibit a smaller, round cell shape, a characteristic phenotype of starved cells. This may imply that Δ*int6* cell is defective in endocytosis and cannot uptake components necessary for recovery from a saturated state.

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