A Novel Fission Yeast Gene, \textit{tht1}+, Is Required for the Fusion of Nuclear Envelopes during Karyogamy

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Abstract. We have isolated a fission yeast karyogamy mutant, \textit{tht1}, in which nuclear congression and the association of two spindle pole bodies occurs but the subsequent fusion of nuclear envelopes is blocked. The \textit{tht1} mutation does not prevent meiosis, so cells execute meiosis with two unfused nuclei, leading to the production of aberrant ascus. The \textit{tht1} gene was cloned and sequenced. Predicted amino acid sequence has no significant homology to previously known proteins but strongly suggests that it is a type I membrane protein. The \textit{tht1} gene is dispensable for vegetative growth and expressed only in conjugating cells. Th1p is a glycoprotein susceptible to endoglycosidase H digestion. Site-directed mutagenesis showed that the N-glycosylation site, as well as the COOH-terminal region of Th1p, is essential for its function. A protease protection assay indicated that the COOH terminus is cytoplasmic. Immunocytological analysis using a HA-tagged Th1p suggested that the protein is localized in nuclear envelopes and in the ER during karyogamy and that its levels are reduced in cells containing fused nuclei.

Karyogamy, or nuclear fusion, is a process in which two haploid nuclei fuse to produce a diploid nucleus in yeast. Genetic and cytological analyses in \textit{Saccharomyces cerevisiae} showed that karyogamy consists of at least two distinct processes: one is the congression of two nuclei and the other is the fusion of nuclear envelopes of juxtaposed nuclei (Rose, 1991; Kurihara et al., 1994). The mixing of chromosomes and nuclear matrices brought by the two nuclei must follow envelope fusion and results in the formation of a diploid nucleus, however, this aspect of karyogamy has received little attention to date. Genes identified in \textit{S. cerevisiae} as required for the nuclear congression include \textit{KAR1}, \textit{KAR3}, \textit{CIK1}, \textit{BIK1}, and \textit{TUB2}, which are all components of spindle pole body (SPB)–microtubular system (Rose, 1991). These factors are also shown to be either essential or important for vegetative growth. \textit{KAR4} is involved in the expression of \textit{KAR3} and \textit{CIK1} and therefore also required for the nuclear congression (Kurihara et al., 1996). On the other hand, a class of endoplasmic reticulum proteins, such as Kar2p (BiP), Sec63p, Sec71p, Sec72p, and Jem1p (Normington et al., 1989; Rose et al., 1989; Ng and Walter, 1996; Nishikawa and Endo, 1997), were all shown to be required for the fusion of nuclear envelopes. BiP is known to be a ubiquitous ER luminal protein which is a member of stress-inducible chaperones. BiP functions in modulating protein folding and protein translocation (Gething and Sambrook, 1992). Sec63p, Sec71p and Sec72p form a membrane-bound complex required for protein translocation (Deshaies et al., 1991; Green et al., 1992; Ng and Walter, 1996), and Kar2p has been shown to interact with Sec63p (Scidmore et al., 1993; Brodsky et al., 1995). However, it remains to be elucidated how these ER proteins are involved in the nuclear envelope fusion.

We have been interested in the karyogamy in \textit{Schizosaccharomyces pombe}, because cytological studies have proven it to be an excellent model system to study the regulation of nuclear organization. Very dynamic yet genetically regulated rearrangement of chromosomes, as well as morphological changes in nuclear shape, can be observed upon entry into and during karyogamy and subsequent meiosis in the fission yeast. In vegetatively proliferating cells, centromeres are either located near the SPB as a cluster or linked to the SPB with microtubules. Thus, the SPB take a major role in the positioning and movement of chromosomes (Funabiki et al., 1993). In sharp contrast to the situation in the mitotic cell cycle, during karyogamy and meiotic prophase telomeres form a single cluster near the SPB and dominate in the chromosomal movement (Chikashige...
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et al., 1994, 1997). The SPB appears to play a vital role not only in these chromosomal events but also in the movement of nucleus, that is, during both congression of two haploid nuclei and the oscillatory movement of a meiotic prophase nucleus along the long axis of the zygote, the SPB leads the nuclear movement (Chikashige et al., 1994). The nucleus in the meiotic prophase is also known to have characteristically elongated and ever changing morphology, often referred to as a “horse tail” nucleus (Robinow, 1977).

We have recently isolated a fission yeast mutant, \textit{kms1} (Shimanuki et al., 1997), which does not form the smoothly elongated horse tail nucleus in meiotic prophase but produces an aberrantly shaped nucleus in which the telомерes often fail to form a single cluster. Instead they tend to be distributed in multiple clusters. The \textit{kms1} mutant is also impaired in karyogamy and in the meiotic chromosome segregation and sporulation. These mutant phenotypes are most likely correlated with the failure of SPB function. In fact, we recently found that the \textit{kms1} gene product may function as an indispensable component of the SPB when the telomere cluster associates with it (Shimanuki, M., unpublished observation). The observations of the \textit{kms1} phenotype further emphasized the importance of SPB function and chromosomal organization in sexual nuclear processes in fission yeast. In an effort to identify more factors involved in such a process, we previously made a collection of mutants that were defective in diploid formation after conjugation (Tange and Niwa, 1995). In this study we have isolated and characterized a mutant that is defective in the \textit{tht1} gene. Results described in this report indicate that the nuclear congression and the association of two SPBs takes place but that the subsequent fusion of nuclear envelopes is blocked in the mutant. The \textit{tht1} gene encodes a novel glycoprotein transiently produced during conjugation. It is the first gene identified in the fission yeast that may be specifically required for the fusion of nuclear membranes during karyogamy.

**Materials and Methods**

**Genetic Procedures**

Standard genetic methods were followed (Gutz et al., 1974; Moreno et al., 1990). YEAY and YPD were used as complete media, MEA for conjugation and sporulation, and EMM2 as a minimal medium. In some experiments conjugation was induced in liquid according to the procedure described by Miyata et al. (1997). In brief, heterothallic strains of different mating types were cultured separately at 30°C overnight to a concentration of 1 \times 10^6 cells/ml in MEB-Gal medium (2% malt extract [Oxoid] and 1% D-galactose). For the nitrogen starvation, cells were washed once in 0.1% D-glucose and the two strains were mixed in MSM medium (1% D-galactose, 0.4% D-mannose, 0.15% K,HPO, and four vitamins as in EMM2), each at a concentration of 3 \times 10^6 cells/ml, followed by incubation at 30°C with vigorous shaking (140 rpm). For homothallic strains the final cell density in MSM was brought to 6 \times 10^7 cells/ml. In the experiment for immunolocalization, we induced conjugation by the procedure described by Beach et al. (1985).

**Mutant Isolation**

We have made a mutant collection based on the genetic background of \textit{h^6 nei1-102 leu1 ts1}, aiming a class of mutants defective in the diploid formation after conjugation (Tange and Niwa, 1995). Individual strains were examined for the nuclear morphology in zygotes by 4,6-diamidino-2-phe- nylinolide (DAPI) staining. Strain DF4-3 found in this screening was subjected to detailed analyses in this study.

**Determination of Ploidy**

Homothallic yeast strains were incubated on a MEA medium at 30°C overnight to induce zygote formation. Individual zygoes were transferred onto fresh YEAY medium using a micromanipulator and incubated at 30°C for several days. Colonies produced from the separated zygoes were examined for their ploidy by the flow cytometric analysis as described in Tange and Niwa (1995).

**Plasmids**

The multicopy plasmid pKD10 (Shimanuki et al., 1997) was used for subcloning and complementation test. Fragments inserted into the vector are shown in Fig. 6A. For the overexpression experiments, pREP1, pREP42 (gifts from D. Maundrell, Glaxo Institute for Molecular Biology, Geneva, Switzerland) and pAS248 (Toda et al., 1991) were used. pREP41 and pREP42 carry the \textit{nrml}-promoter (Maundrell, 1993), which is inducible by thiamine depletion. A DNA fragment covering the \textit{tht1} ORF was produced by PCR and inserted at the NdeI-BamHI site of pREP1 and pREP42 to make pNT45 and pNT79, respectively. pAS248 carries a constitutively active \textit{ath} promoter. The BamHI-PsiI fragment from pNT37 was blunt ended and inserted into the Smal site in pAS248 to generate pNT79. The HA-tag sequence (\textit{AHPDVPDYA0YPDVPDYA0MGY- PYPDVPDYA}), repeated HA-epitopes are underlined) was amplified from a plasmid pHA4 (a gift from I. Hagan, University of Manchester, Manchester, UK) and inserted at the BamHI, HpaI, Stul, and NcoI sites in pNT28 to make pNT72, pNT73, pNT74, and pNT75, respectively. pNT72, pNT73, and pNT74 could not complement the \textit{tht1}-mutant, whereas pNT75 could. A control plasmid, pNT77, containing the HA-tag sequence was constructed by replacing the NspV fragment of pNT28 with the HA-tag sequence. In this construct the HA-tag is fused to the NH2-terminal 38 amino acids of \textit{tht1}. The plasmid pEB9 carries the BiP gene tagged with the myc epitope (Pidoux and Armstrong, 1992). The plasmid D817, which was employed for staining the nuclear envelope in this study, had been isolated from a GFP-fusion genomic library based on a monocopy vector with the LUE2 selection marker. Sequence determination of the D817 clone showed that the 276 amino terminal residues of the 678-amino acid cytochrome P-450 reductase (Miles, 1992) were fused with GFP in this plasmid. D-2, and Y. Hiraoka, manuscript in preparation). GFP-tagged Kms1p was used to stain the SPB, which was expressed from the pGK77, a pREP1-based plasmid. The fusion protein produced under the repressive condition was shown to localize to the SPB. Details about the localization of Kms1p will be published elsewhere.

**Gene Cloning and Sequencing**

\textit{tht1} gene was cloned from cosmide libraries of \textit{S. pombe} genomic DNA. One of the libraries had been made by using a cosmid vector scos1-LEU2 (Murakami and Niwa, 1995). Another library was obtained from Dr. D. Beach via Dr. Y. Oshima (Kyushu University, Japan), which is based on the pSS10 vector (Nakaseko et al., 1986). \textit{h^6 leu1-32 tht1-1} was transformed into the lithium acetate method. Cosmid DNA was recovered from yeast transformants according to Moreno et al. (1991). One cosmid, cos737, was used for further study. The relevant DNA segments were sequenced by the dideoxy method. cDNA analysis was performed as described in Shimanuki et al. (1997).

**One Step Gene Disruption**

The 2.9-kb SalI fragment of the \textit{tht1} gene was inserted in the Smal site of Bluescript II (Stratagene, La Jolla, CA), and the \textit{ura4} gene sequence was substituted with the 1.7-kb NspV fragment of the insert to generate pNT31. This substitution nearly completely disrupts the \textit{tht1} gene, leaving only 38 amino acid residues at its NH2 terminus. The KpnI-SacI fragment of pNT31 was excised and transferred into a diploid cell to obtain stable transformants, for which Southern blot hybridization was performed to verify the correct integration. For the disruption of the 740-bp open reading frame (ORF), the 2.2-kb NspV fragment bearing the ORF was inserted into the EcoRV site in Bluecript II (pNT60). The EcoRIEcoRV fragment of pNT60 was replaced with the \textit{ura4} sequence to completely remove the ORF sequence and then the XhoI-SpeI fragment was cut out of this plasmid for transformation.

**Northern Blot Hybridization**

Total RNA was extracted according to Alfa et al. (1993). Poly(A)+

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mRNA was purified by using the oligotex-dt30 (super; Takeda Pharmaceutical Co., Osaka, Japan). RNA probes were used for hybridization. Each probe was transcribed from the T7 promoter using the DIG RNA labeling kit (Boehringer Mannheim Corp., Indianapolis, IN) with the following template plasmid DNAs. For probe 1 (see Fig. 8) the XhoI-RsaI fragment (from nucleotide -1202 to -189, numbering is from the initiation codon of the tht1+ gene) was ligated with the XhoI-EcoRV site of BlueScript II to make pNT46. XhoI-digested pNT46 DNA was used as the template. For probes 2 and 3 the HindIII-EcoRV fragment (from nucleotide -1271 to +670) was cloned into the HindIII-SmaI site of pKD10 (pNT25), pNT25 was digested at the Sall site (located at -39). For probes 3, 4, and 5 the EcoRV fragment (+670 to +1588), the MunI fragment (+2030 to +2482, blunt ended), and the Ddel fragment (+2781 to +3333; blunt ended) were inserted into the EcoRV site of BlueScript II in an appropriate orientation. For probe 6 the SalI fragment, pNT59, and then digested with HindIII, HindIII, and XhoI, respectively, before use. Another plasmid, pNT47, was made by ligating the RsaI-HindIII fragment (+2256 to +2922) with the HindII and HindIII sites in BlueScript II followed by digestion with XhoI. This probe was used for the analysis of the disruptant of the 740-bp ORF (see text). Hybridization and washing was conducted using the standard conditions recommended by Boehringer Mannheim Corp., with the exception of probe 4, which was hybridized in a high SDS buffer without formamide to reduce the stringency of hybridization (Church and Gilbert, 1984). Band detection was performed with the DIG luminescent detection system (Boehringer Mannheim Corp.).

Production of Antibodies

A segment of the tht1+ gene corresponding to amino acids 42–154 was inserted into the pGEX vector (Pharmacia Biotech, Inc., Piscataway, NJ) to produce a GST-fused protein. Bacterially produced fusion protein was purified and injected into rabbits to raise antibodies. Antibodies were affinity purified with the fusion protein according to Smith and Fisher (1984).

Preparation of Cell Extracts and Western Immunoblotting

Cell extracts for Western immunoblotting were prepared according to Moreno et al. (1991) and Funabiki et al. (1996) with modifications. 3 x 10^7 cells were harvested and washed once with ice-cold STOP buffer (150 mM NaCl, 50 mM NaF, 10 mM ethylenedinitetraacetate [EDTA], pH 7.5, 10 mM EDTA, 10% glycerol, 30 mM NaCl, 1 mM DTT, 1 mM PMSF, 2 μg/ml peptatin A, 10 μM E-64) and disrupted with 0.3g glass beads (425–600 μm) at 4°C for 5 min to remove insoluble materials. Proteins were run on 10% SDS-PAGE gels and transferred to nitrocellulose membrane. Protein detection was performed by chemiluminescence detection system (ECL; Amersham Corp., Arlington Heights, IL) with horse radish peroxidase-conjugated protein A (Amersham Corp.).

Overexpression of the tht1+ Gene

For the overexpression of the tht1+ gene in vegetative cells, HM123 (h-leu1) was transformed either with pNT45 or with pNT79. Transformants carrying pNT45 were transferred from EMM2 with thiamine to the thiamine-depleted medium at the cell density of 3 x 10^7 cells/ml at 30°C to induce the expression of the tht1+ gene.

Digestion with Endoglycosidase H

Cell extracts prepared by the alkaline lysis method were digested with endoglycosidase H (endoH; New England Biolabs Inc., Beverly, MA) according to the manufacturer's instructions.

Site-directed Mutagenesis

The Quikchange site-directed mutagenesis kit (Stratagene) was used to introduce desired mutations in pNT28. For each mutant gene the entire nucleotide sequence of the ORF was determined to confirm that only the desired changes had been introduced.

Fluorescent Staining

For indirect immunofluorescent staining, we followed the procedure described in Chappell and Warren (1989) but Novozyme was omitted from the digestion mixture. In this method, 4% formaldehyde was used for fixing cells but glutaraldehyde was not included. In some experiments, including GFP-DAPI double staining, the method described in Shimanuki et al. (1997) was followed. But for double staining, cell wall digestion was performed only briefly and the Triton X-100 treatment was omitted. In some experiments, including GFP-DAPI double staining, the method described in Shimanuki et al. (1997) was followed. But for double staining, cell wall digestion was performed only briefly and the Triton X-100 treatment was omitted. Mouse monoclonal anti–HA antibody (12CA5; Boehringer Mannheim Corp.) was used in combination with a goat anti-mouse Cy3-conjugated antibody (The Jackson Laboratories, Bar Harbor, ME). We also used a goat anti-rabbit Cy3-conjugated antibody (Jackson Laboratories). For immunolocalization of the tagged BIP, mouse monoclonal anti–e–y antibody (9E10; Sigma) was used (Pidoix and Armstrong, 1992). For membrane staining 0.25 or 0.125 μg/ml of DIOC(3) (3,3-diethylxocarboxylic acid iodide; LAMBDA, Graz, Austria) was added (Terasaki, 1994). Fluorescent in situ hybridization (FISH) was performed using a telomere specific probe cos212 according to Shimanuki et al. (1997). Microscopic observation was carried out with the Delta Vision system (Applied Precision Inc., Issaquah, WA). Coloring in all figures was arbitrary.

Results

tht1 Mutant Is Defective in the Nuclear Fusion

From a collection of S. pombe mutants that are defective in diploid formation after mating, we found one in which nuclear fusion was severely impaired. Tetrad analysis showed...
that this defect was due to a mutation in a single nuclear gene, which we have designated tht1 (twin horse tails, see below). Homothallic (self conjugating) strains with or without the mutation were compared for the efficiency of nuclear fusion by DAPI staining. These strains carried the mei1-102 mutation that blocks meiotic divisions after nuclear fusion (Egel, 1989), so that 99% of the tht1 mutants contained a single fused nucleus (Fig. 1a; (b and c) tht1-1; (d and e) mei1+ zygotes in the horse tail stage; (d) tht1+; (e) tht1-1. Bar, 5 μm.

while maintaining their individual identity, suggesting that the unfused state is fairly stable. Consistent with this, when we put individual zygotes of the tht1 mei1 double mutants back into nutrient-rich medium to let them reenter the cell cycle and form colonies, ~85% were found to form haploid colonies. Under the same condition, tht1+ zygotes produced diploid colonies in >90% of the cases.

**Twin Horse Tails in the tht1 Mutant**

Wild-type *S. pombe* cells usually proceed to meiosis immediately after nuclear fusion (zygotic meiosis), although meiosis can be induced directly from a diploid cell without intervening conjugation (azygotic meiosis). In both of these types of meiosis, prophase is accompanied by the presence of an oscillating horse tail nucleus (see Introduction, Fig. 1d). When the tht1 mutant was induced to undergo a zygotic meiosis, we did not find a single horse tail nucleus in each cell, but instead we frequently found a pair of small horse tail nuclei lying side by side both by DAPI

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**Figure 1.** The terminal phenotype of the tht1-1 mutant. Strains carrying D817 plasmid were incubated on MEA at 18°C for 3 d. (Left) Stained with DAPI; (right) nuclear envelopes visualized with a GFP-fused hybrid protein produced from D817 (see text). (a–c) zygotes with mei1-102 mutation, blocking before entry into meiosis. (a) tht1+; (b and c) tht1-1; (d and e) mei1+ zygotes in the horse tail stage; (d) tht1+; (e) tht1-1. Bar, 5 μm.

**Figure 2.** (A) FISH analysis of the twin horse tails with a telomere probe (right). (Left) Merged image of DAPI (light blue) and telomere-FISH (red). (B) SPBs stained with the anti–Sad1 antibody (right). Merged images of (light blue) DAPI stains and (red) anti–Sad1 (left). Bar, 5 μm.
staining and by the nuclear envelope staining (Fig. 1 e). As anticipated, we found that telomere clusters were located near the tips of the paired nuclei (Fig. 2 A). In these twin horse tails the pointed ends were always on the same side, suggesting that these pairs of horse tail nuclei were moving in a coordinated fashion. In fact, when we observed the horse tail movement in live cells according to the procedures of Chikashige et al. (1994), twin horse tail nuclei always moved in such a way that the two pointed ends were dragged together from a single oscillating site where the SPB should reside, suggesting that in the conjugation of the tht1 mutant the pair of SPBs have bound to form a single diploid SPB after the nuclear congression, which can lead the movement of two unfused horse tail nuclei. To verify this, we performed an immunofluorescence staining of the SPB using the anti–Sad1 antibody (Hagan and Yanaida, 1995). In this analysis, we have carried out optical sectioning with 0.1-μm intervals to minimize the possibility of overlapped signals. In 24 out of 25 cases observed, only a single stained spot could be observed (Fig. 2 B). In the remaining one case, two distinct spots were observed, although they were very closely opposed. In wild-type control cells, in all of 25 cells single spot was stained. Similar results were obtained from analysis of a tht1 mutant strain with the mei1 mutation. In this analysis, GFP-tagged Kms1p was used for staining the SPB, because this fusion protein had been shown to localize to the SPB (Shimanuki, M., unpublished result). In 22 out of 25 cases, there was only one signal between juxtaposed nuclei (Fig. 3) and in the remaining three cases two adjoining spots were observed. In a tht1+ control strain, 24 out of 25 contained single spots. These observations not only confirmed the results on the twin horse tail nuclei, but also indicated that the binding or close association of SPBs was completed before the horse tail period, that is, meiotic prophase.

**Aberrant Sporulation in tht1 Zygotes**

Zygotic asci produced by the tht1 mutant were abnormal, containing an irregular number of spores with aberrant morphology (Fig. 4). DAPI-stained bodies observed in these mutant asci were also irregular in number and intensity. Furthermore, they often contained nuclei that were not encapsulated with spore walls (Fig. 5, b, e, f, arrows). The viability of the spores, which were released by enzymatic treatment of the asci, was 28%, while for wild-type control spores it was 70%. Moreover, as many as 35% of the viable mutant spores were diploid. These results suggested that chromosomes were not correctly transmitted into the mutant spores. When diploid cells homozygous for the tht1 mutation were induced to carry out meiosis/sporulation, however, resultant azygotic asci appeared completely normal and contained four viable haploid spores (Fig. 4). The recombination frequency as well as the morphology of horse tail nucleus was also normal in the azygotic meiosis (data not shown), indicating that the tht1 mutation itself does not interfere with meiosis and sporulation. Therefore, it is likely that the aberrant mutant asci were produced via the inability to fuse the nuclei resulting in the twin horse tail stage.

**Cloning of the tht1+ Gene**

Since the tht1 mutation was recessive to wild type regarding the abnormal ascus formation, the tht1+ gene was cloned by using cosmid libraries. Two overlapping cosmids derived from the same chromosomal region were isolated. Integration mapping indicated that the cosmid clones contained the tht1+ gene. By subcloning and sequencing segments of the cosmids we found that the tht1+ gene contained an ORF of ~1.8 kb that was interrupted with two introns. The presence of these introns has been confirmed by a PCR based cDNA analysis. We found that a cosm id sequence that had been previously deposited in the EMBL/GenBank/DDBJ database (available under accession num-
ber Z50112) contained the tht1+ sequence. This cosmid (c13C5) had been mapped to the distal portion of the left arm of chromosome I (Hoheisel et al., 1993). We then made a complete loss-of-function mutant allele at the tht1 locus by a one-step gene disruption method (Materials and Methods). Strains carrying the disruptant allele were viable and did not show any defective phenotype in vegetative growth, but, as anticipated, were as defective in karyogamy as the original tht1-1 mutant.

The predicted amino acid sequence of Tht1p along with a hydropathy plot is shown in Fig. 6. The calculated mol wt of the 577–amino acid protein is 66.9 kD. Computational searches have failed to find out any known proteins with significant sequence homology, indicating that Tht1p represents a novel class of proteins. There are three potential transmembrane segments in the COOH-terminal half of the protein, with positively charged amino acid residues enriched between segments 1 and 2 and near the COOH-terminal side of segment 3 (Fig. 6). There is also a typical signal peptide sequence at the NH2 terminus. These features of the protein may indicate that the tht1+ gene product is a type I membrane protein, with the COOH-terminal portion being in the cytoplasm (High and Dobberstein, 1992).

The Expression of tht1+ Gene Is Confined to Conjugating Cells

Northern blot hybridization analysis was performed using probes specific for the tht1+ gene (Fig. 7). About 3 h after the induction of conjugation by nitrogen starvation, two kinds of poly(A)+ transcripts of ~1.8 and 3.8 kb appeared and the amount of these transcripts peaked at 5 h and then decreased (Fig. 7 A). The timing of the induction was roughly coincident with the appearance of copulating cells. Nitrogen starvation and the presence of two different mating type genes were not sufficient for the induction, since neither heterothallic haploid cells nor diploid cells heterozygous for the mating type gene could produce the transcripts after nitrogen starvation (Fig. 7 B). These results indicated that tht1 gene is only expressed in conjugating cells.

We then examined the origins of the two different tran-
scripts. Southern hybridization analysis indicated that there is only one copy of the tht1^{+} gene in the genome. We performed Northern hybridization using several different probes specific for either the tht1^{+}-ORF sequence or neighboring sequences (Fig. 8). Both 1.8- and 3.8-kb mRNAs were hybridized to probes 2 and 3, which were specific for 5' and 3'-half of the ORF, respectively. However, with probes 4 and 5, only a 3.8-kb band was detected. Interestingly, probe 5 also hybridized to a 0.8-kb mRNA, which was probably corresponded to the 740-bp ORF. Probe 1, an upstream specific probe, did not hybridize to the tht1^{+} transcripts, but instead hybridized to a 1-kb mRNA covering the 1-kb ORF. A PCR-based analysis suggested that both of the two transcripts start at about the same site (~50 bp upstream from the initiation codon) and that the 1.8-kb mRNA terminates ~100 bp downstream from the termination codon. These results indicated that the tht1-ORF is transcribed with two different mRNAs. Since it was possible that the 740-bp ORF has some functional relation to the tht1 gene, we disrupted this ORF by a ura4^{+} substitution method (Materials and Methods). The disruptant was viable and displayed no karyogamy related phenotype. In this disruptant, the 0.8-kb mRNA was no longer transcribed and the 3.8-kb mRNA became shorter. The amount of the 1.8-kb mRNA in the disruptant was comparable to its level in wild-type controls (data not shown).

**Identification of the tht1^{+} Gene Product**

We prepared extracts from cells carrying a multicopy plasmid with the tht1^{+} gene (pNT28) and performed a Western immunoblot analysis using anti–Tht1p antibodies (1-3-2). We found a 67-kD protein specifically expressed in conjugating cells (Fig. 9 A). The temporal expression profile was very similar to the mRNA accumulation profiles detected by Northern hybridization analysis. Moreover, the protein levels were very low in strains without the plasmid or with the vector alone (Fig. 9 A), suggesting that the 67-kD band represented the tht1^{+} gene product. This protein partitioned to the insoluble fraction (Fig. 9 B). It could barely solubilized with 1% Triton X-100 or with 2 M NaCl (data not shown). The same 67-kD insoluble protein was produced when we forced gene expression in mitotic cells by using the adh-promoter (see Fig. 9 C).

**Tht1p Is a Glycoprotein**

Since there are two potential N-linked glycosylation sites in the tht1^{+} protein sequence, we examined whether Tht1p is actually glycosylated. To this end we took two approaches. First, we examined whether the protein is a sub-
strate for endoH, an N-glycosylation–specific glycosilase. Results shown in Fig. 9 indicated that endoH digestion could make the 67-kD band shift down to 64 kD. This 3-kD difference roughly corresponds to the removal of a single oligosaccharide. Second, we performed site-directed mutagenesis to change the acceptor Asn residues to Asp. Mutant genes carrying N163D, N372D, or doubly mutated sequence were expressed in conjugating cells. Western immunoblot analysis clearly indicated Asn163 is glycosylated, but that Asn372 is not a target of N-glycosylation (Fig. 9D).

Functional complementation analysis showed that neither the N163D mutant nor the double mutant were able to rescue the tht1-1 mutation, while N372D was as active as wild type (Fig. 10). From these results we concluded that the Asp residue at 163 is glycosylated and that this modification is likely to be essential for function.

The COOH-terminal End of Tht1p Is Essential and Cytoplasmic

In the original tht1-1 mutant gene we found a one-base substitution near the 3’-end of the ORF (Fig. 10). It was a nonsense mutation that would produce a truncated protein missing three amino acids (WWD) from the COOH terminus. This suggested the COOH-terminal end of the protein performs an essential functional role. To test this possibility we created several kinds of mutations near the COOH terminus. For each of these mutant genes we examined the ability to rescue the defects both in nuclear fusion and ascus formation of a strain carrying the tht1 disruption allele. Results summarized in Fig. 10 indicated that both of the two consecutive tryptophane residues are essential for function. Alterations in the surrounding amino acids had very little effect except for the deletion of the most terminal Asp residue which led to a partial loss of function. Even the addition of an extra sequence of four amino acids to the COOH terminus, as well as the insertion of the HA-tag sequence before the PWWD sequence, did not impair the activity. It should be noted that Western blot analysis showed that the amount of protein produced from each of these mutant genes was comparable to that produced from the wild-type gene (data not shown).

As noted above, it was predicted from the amino acid sequence that the tht11 gene product is a type I membrane protein, with its COOH terminus in the cytoplasm. To test this prediction, we prepared microsomes from conjugating cells expressing HA-tagged or wild-type Tht1p. One of the tagged proteins, Tht1-NcoI-HA, bears the HA epitope near the COOH terminus, while the other one, Tht1-StuI-HA, contained it on the NH2-terminal side of the presumed membrane-spanning domain. When the microsome samples were incubated with trypsin and subjected to Western immunoblot analysis using anti–HA antibodies, the band intensity of Tht1-NcoI-HA fusion decreased as the amount of trypsin increased (Fig. 11A, lanes 1–5). However, when Tht1-StuI-HA was examined there was a shift to a band that migrated with a mobility equivalent to a reduction in size of 3 kD and there was only a partial decrease in band intensity. (Fig. 11A, lanes 11–15), indicating that the HA sequence was more resistant to trypsin in Tht1-StuI-HA than in Tht1-NcoI-HA. The 55-kD band that appeared in the preparation of Tht1-StuI-HA was also resistant to trypsin digestion. This band probably corresponded to a polypeptide truncated near the COOH-terminal end of the third transmembrane segment. When trypsin digestion was performed in the presence of Triton X-100, which can permeabilize microsomes, the bands from Tht1-StuI-HA were no longer resistant to trypsin (Fig. 11A, lanes 16–20). We
then used anti–Tht1p antibody 1-3-2, which was raised against a segment from the NH₂-terminal part of Tht1p, to probe the tryptic digests. Upon digestion of Tht1-NcoI-HA by trypsin, as the intensity of the 76-kD band decreased, a lower 64-kD band became more intense (Fig. 11B, lanes 1–3). The same 64-kD band was produced from Tht1p in the absence of Triton X-100 (Fig. 11B, lanes 13–15). In the presence of the detergent, this 64-kD band was not stable in either Tht1-NcoI-HA or Tht1p (Fig. 11B, lanes 4–6 and 16–18). These results were all consistent with the notion that the COOH terminus of Tht1p is in the cytoplasm and the NH₂-terminal portion is in the lumen so that it is protected from attack by trypsin in these assays.

**Tht1p Is Localized in Nuclear Envelope and ER during Karyogamy**

To determine the location of Tht1 protein, we first attempted to immunostain wild-type cells or those carrying integrated HA-tagged tht1 gene, but we failed to observe significant signals from these cells. We then expressed an HA-tagged Tht1 protein (Tht1-NcoI-HA) from a multicopy plasmid. This construct (pNT75) was capable of complementing the tht1 deficiency (see Fig. 10). Staining with anti–HA antibodies revealed signals from the nuclear peripheries in some fraction of the conjugating cells. In these cells there were always some aggregate-like bright signals in cytoplasm (Fig. 12, a and b). In other words, we encountered very few cells in which only the nuclear peripheries were stained. When the same cells were simultaneously stained with a general membrane dye, DiOC₆(3), we found that the regions where the anti–HA antibodies stained were also stained by the dye. With DiOC₆(3), however, more cytoplasmic components were stained and brighter signals were produced. In general DiOC₆(3) staining was not coincident with the anti–HA signals (see Fig. 12). Interestingly, when cells had formed single fused nuclei, we could no longer see the anti–HA signal from the nuclear peripheries and the signals in cytoplasm were also diminished (Fig. 12c). Another HA-tagged protein (Tht1-StuI-HA; pNT74) was examined for its localization. This protein was also localized around the nuclei and in the amorphous cytoplasmic components, however, the signal intensity was more concentrated around the nuclear peripheries and was at a much-reduced level in the cytoplasm when compared to Tht1-NcoI-HA (Fig. 12d). It should be noted...
that pNT74 was unable to complement the tht1 mutation (Fig. 10). At the present, we do not understand the apparent difference in the signal distribution. We also do not know which cytoplasmic components were stained with the anti–HA probe, although they are likely to represent ER. Control cells carrying pNT77 (Nter-Tht1-HA) displayed no specifically localized anti–HA signals, although the DiOC₆(3)-staining images were indistinguishable from played no specifically localized anti–HA signals, although this fixative may not be suited for the preservation of membranous structure (Terasaki, 1994). However, the glutaraldehyde fixation method could be applied to the experimental cells (Fig. 12). It should be noted that we first attempted to use glutaraldehyde to fix cells but we found this method produced very high background. Therefore we employed formaldehyde for subsequent fixations, although this fixative may not be suited for the preservation of membranous structure (Terasaki, 1994). However, the glutaraldehyde fixation method could be applied to vegetative cells. We have overproduced Tht1p by using the nmt1+ promoter, which can be induced by thiamine depletion (Maundrell, 1993), and stained the cells with anti–Tht1p antibodies 1-3-2. As shown in Fig. 13 b, 14.5 h after the induction, we could find bright signals around the nucleus as well as in cytoplasmic reticular structures and the periphery of cytoplasm. While in cells harvested 10 h after induction, when only low levels of Tht1p could be detected by Western immunoblot analysis, very few such bright signals could be observed (Fig. 13 a). When we overproduced Tht1p by using a constitutively active promoter, the adh promoter, which has been shown to be less active than the nmt1+ promoter (Basi et al., 1993), we could observe bright signals only from the nuclear peripheries, and signals in the cytoplasm were weaker than the cases when the nmt1+ promoter was used (Fig. 13 e). The location of Tht1p in these vegetative cells was reminiscent of the distribution of BiP, an authentic ER protein, in S. pombe (Pidoux and Armstrong, 1992, 1993). Consistently the distribution of BiP and Tht1p in cells overexpressing both proteins was virtually identical (Fig. 13, d and e).

Discussion

The tht1+ gene reported in this study is the first karyogamy-specific gene identified in S. pombe. The kms1+ gene, which we identified recently, also plays an important role in karyogamy but it is required for progression through meiosis as well, particularly in the formation of the meiotic prophase-specific nuclear architecture (Shimanuki et al., 1997). The tht1 mutant severely blocks nuclear fusion, but does not affect meiosis at all when meiosis is induced directly from a diploid cell. We also showed that tht1 gene is dispensable for mitotic growth by completely disrupting the gene. In accordance with these observations, the expression of tht1+ gene is strictly confined to conjugating cells, where the gene product is required. The fission yeast gene fus1+ is also specifically required for conjugation but at an earlier step before karyogamy, that is, the degradation of cell walls leading to cytoplasmic fusion after the cell contact. It has been reported that the expression of fus1+ gene is dependent upon nitrogen starvation and the presence of cells of the opposite mating type (Petersen et al., 1995), a condition similar to that for the tht1 gene expression. It will be interesting to see if there are a class of genes specifically involved in conjugation and/or karyogamy that are under the same genetic control.

Aberrant Meiotic Divisions from Unfused Nuclei

In fission yeast meiosis, like in mitosis, the spindle is formed between separated SPBs in the nucleus while keeping nuclear envelope intact (Hirata and Tanaka, 1982). Before the SPB proceeds into the duplication/separation cycle in meiosis, the two haploid SPBs must fuse to make a single unified SPB in karyogamy. A couple of pieces of evidence shown in this study suggest that the SPBs have completed this binding process before the tht1 arrest point. First, in almost all of the cases, only one SPB signal could be observed between two juxtaposed nuclei using the SPB-specific probes, anti–Sad1 antibodies and GFP-fused Kms1p. A second piece of evidence is provided by the coordinated movement of the twin horse tail nuclei, where only a single SPB seems to lead the movement. However, we have not ruled out the possibility that the binding of the SPBs is incomplete in the mutant. Rather, we think it

Figure 13. Localization of overproduced Tht1p. (a and b) HM123 (h+ wild type) transformed with pNT45 was induced by thiamine depletion for 10 h (a) and 14.5 h (b). (c) HM123 was transformed with pNT79. Anti–Tht1 antibody 1-3-2 was used to stain for Tht1p. (d and e) HM180 (h- leu1 ura4) was transformed with both pEB9 and pNT79 and incubated for 14 h in the absence of thiamine. Cells were doubly stained with anti–c-myc 10E9 and anti–Tht1p antibody 1-3-2. Bar, 5 μm.
could be a considerable possibility, because there could be strong tension produced by such unusual configuration of unfused and bulky nuclei against the SPB. In any case, there must be a topological problem in the formation of the spindle in a mutant situation such as that seen in \textit{tht1} where one unified SPB or two separating SPBs have to be stuck to the two unfused nuclear envelopes. In fact, we could find very few normal spindles and DAPI-stained images of separating nuclei in the mutant zygotes (data not shown). Although we could not strictly rule out the possibility that meiotic division is prohibited from the twin horse tail state, we feel that it is unlikely judging from the efficiency of sporulation. Moreover, the \textit{twsl} mutation that blocks meiosis II (Nakaseko et al., 1984), when combined with the \textit{tht1} mutation, reduced the number of spores (data not shown), indicating that even in the aberrant meiosis/sporulation in the \textit{tht1} mutant, meiosis II, and therefore meiosis I as well, does take place. In this regard we thought it interesting that a considerable number of mutant asci contained a large nucleus that was not encapsulated with the spore wall (Fig. 5, arrows). Since it is known that spore wall formation initiates from a modified SPB (Tanaka and Hirata, 1982), we first speculated that this type of nucleus might have arisen from an event where the SPB had been stripped off from one of the unfused nuclei. However, to our surprise, such nuclei in fact bore two or three anti–Sad1 stains (data not shown). This phenotype was very unusual and we have currently no plausible explanation for it. Nevertheless, it seems apparent that the SPB-spindle cycle became catastrophic in the mutant zygotylic meiosis. It may be also worth considering here that the SPB had been stripped off from one of the unfused nuclei. In any case, we felt that it is unlikely judging from the efficiency of sporulation. Moreover, the \textit{twsl} mutation that blocks meiosis II (Nakaseko et al., 1984), when combined with the \textit{tht1} mutation, reduced the number of spores (data not shown), indicating that even in the aberrant meiosis/sporulation in the \textit{tht1} mutant, meiosis II, and therefore meiosis I as well, does take place. In this regard we thought it interesting that a considerable number of mutant asci contained a large nucleus that was not encapsulated with the spore wall (Fig. 5, arrows). Since it is known that spore wall formation initiates from a modified SPB (Tanaka and Hirata, 1982), we first speculated that this type of nucleus might have arisen from an event where the SPB had been stripped off from one of the unfused nuclei. However, to our surprise, such nuclei in fact bore two or three anti–Sad1 stains (data not shown). This phenotype was very unusual and we have currently no plausible explanation for it. Nevertheless, it seems apparent that the SPB-spindle cycle became catastrophic in the mutant zygotylic meiosis. It may be also worth considering here that the SPB had been stripped off from one of the unfused nuclei.

\textbf{The Role of Tht1p in Karyogamy}

During conjugation in the \textit{tht1} mutant, the juxtaposition of two nuclei is not followed by the fusion of nuclear envelopes. Mutants showing similar defective phenotype have been isolated in \textit{S. cerevisiae}, those include \textit{kar2}, \textit{kar5}, \textit{kar7}, \textit{kar8}, sec63, sec71, sec72, and \textit{jem1} (Kurihara et al., 1994; Ng and Walter, 1996; Nishikawa and Endo, 1997). For some of these genes their products have been identified, but very little is known about how these genes are involved in nuclear fusion. For instance, Sec63p, Sec71p, Sec72p, and also Kar2p (BiP) are involved as a complex in protein translocation across the ER membrane (Deshaias et al., 1991; Green et al., 1992; Brodsky et al., 1995; Ng and Walter, 1996), but it has been shown that the activity required for protein translocation is not directly required for the fusion of nuclear envelopes (Ng and Walter, 1996).

The ER is contiguous with the nuclear membrane and so it has not been determined from which site(s) membrane fusion starts in karyogamy. However, it was demonstrated that nuclear fusion is roughly paralleled with the homotypic fusion of ER membranes (Latterich and Schekman, 1994). Mutants defective in the fusion of nuclear envelopes were also defective in the ER membrane fusion both in vivo and in vitro (Kurihara et al., 1994; Latterich and Schekman, 1994). In this respect it is interesting that Cdc48p, an ER protein with homology to Sec18p, has been shown to participate in the fusion of ER membranes (Latterich et al., 1995). Sec18p is a yeast homologue of \textit{N-ethylmaleimide}-sensitive factor (NSF), which together with Sec17p (a homologue of soluble NSF attachment protein \textalpha{SNAP}) is required for vesicular docking. Thus, it appears that Cdc48p is a component of fusion/docking machinery, mainly functioning in the fusion of ER membranes. Whether Cdc48p is required for nuclear fusion is yet to be examined experimentally. Furthermore, it has been shown that some novel proteins which are tightly bound to ER membrane are required for the membrane fusion and that Kar2p may participate in the activation of these proteins (Latterich and Schekman, 1994).

At the present we can not speculate as to the function of Tht1p from its amino acid sequence because of the lack of homology to known proteins. We cannot strictly rule out the possibility that the absence of the fusion of nuclear envelopes is a secondary effect of incomplete SPB binding, although it is unlikely as discussed above. However, several lines of evidence described in this study strongly suggest that Tht1p is a novel ER protein. The primary amino acid sequence indicates that it is a type I membrane protein. Tht1p is actually modified with N-linked glycosylation, and partitions to the insoluble fraction. Finally, immunofluorescence microscopy localized the protein in nuclear envelope and some portion of cytoplasmic membranous structures. In vegetative cells containing overproduced Tht1p it could localize to ER. Taking this fact into consideration together with the observation that the \textit{tht1} mutation is transiently expressed only in conjugating cells, we suggest that Tht1p may be directly and specifically involved in the fusion of nuclear envelopes and the ER membranes during karyogamy. One of the key issues in further studies for elucidating the role of Tht1p in karyogamy may be the role of the COOH-terminal end of Tht1p, because this end of the protein was shown to reside in the cytoplasm and could therefore be interacting with some other factors or perhaps with itself to perform an essential function to achieve the membrane fusion. Searches for such factors are in progress and may help to reveal a novel class of molecules involved in membrane fusion events. Another important issue addressed in this study is the temporal expression of the gene. Particularly interesting is the rapid disappearance of Tht1p from the membranes after the completion of karyogamy. Elucidation of mechanism involved in such elaborate regulation should be an important step toward understanding the membrane dynamics in the cell.

We are grateful to John Armstrong for the BiP plasmid. We thank Iain Hagan for critical reading of the manuscript and valuable comments. We also wish to thank Dr. N. Miyajima for his help in computational analyses.

This work was supported by Grant-in-Aid for Scientific Research on Priority Areas (to Y. Hiraoka and O. Niwa) and by the Kazusa DNA Research Institute Foundation.

Received for publication 14 August 1997 and in revised form 17 November 1997.