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The Fission Yeast *spo14*$^+$ Gene Encoding a Functional Homologue of Budding Yeast Sec12 Is Required for the Development of Forespore Membranes

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The *Schizosaccharomyces pombe spo14-B221* mutant was originally isolated as a sporulation-deficient mutant. However, the *spo14*$^+$ gene is essential for cell viability and growth. *spo14*$^+$ is identical to the previously characterized *stl1*$^+$ gene encoding a putative homologue of *Saccharomyces cerevisiae* Sec12, which is essential for protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus. In the *spo14* mutant cells, ER-like membranes were accumulated beneath the plasma membrane and the ER/Golgi shuttling protein Rer1 remained in the ER. Sec12 is a guanine nucleotide exchange factor for the Sar1 GTPase. Overproduction of *psr1*$^+$ coding for an *S. pombe* Sar1 homologue suppressed both the sporulation defect of *spo14-B221* and cold-sensitive growth of newly isolated *spo14-6* and *spo14-7* mutants. These results indicate that Spo14 is involved in early steps of the protein secretory pathway. The *spo14-B221* allele carries a single nucleotide change in the branch point consensus of the fifth intron, which reduces the abundance of the *spo14* mRNA. During meiosis II, the forespore membrane was initiated near spindle pole bodies; however, subsequent extension of the membrane was arrested before its closure into a sac. We conclude that Spo14 is responsible for the assembly of the forespore membrane by supplying membrane vesicles.

INTRODUCTION

Sporulation, gametogenesis in yeasts, involves two overlapping processes, meiosis and spore formation. Four haploid nuclei produced by meiosis are packaged into individual spores. Spore formation begins with the assembly of a double-layered intracellular membrane, termed the forespore membrane (Yoo *et al.*, 1973). Spore wall material is deposited in the lumenal space of the forespore membrane, and its inner leaflet becomes the plasma membrane of spores. The forespore membrane formation provides a model system for the study of de novo synthesis of membranes within the cytoplasm.

Electron microscopic studies (Yoo *et al.*, 1973; Hirata and Tanaka, 1982; Tanaka and Hirata, 1982; Kishida *et al.*, 1990) and recent fluorescence microscopic observations (Asakawa *et al.*, 2001; Nakamura *et al.*, 2001; Nakase *et al.*, 2001; Nakamura *et al.*, 2002) with the fission yeast *Schizosaccharomyces pombe* have revealed fundamental aspects of forespore membrane formation. The formation of this membrane begins during meiosis II by the fusion of membrane vesicles. The spindle pole body (SPB) in yeast is an equivalent structure to the centrosome in animals and plays a crucial role in the formation of spindle microtubules. The SPB undergoes a morphological change into a multilayered structure before forespore membrane assembly during meiosis II. Using immunofluorescence microscopy, Hagan and Yanagida (1995) observed the alteration of the SPB structure from a dot to a crescent in the corresponding stage of meiosis. Because most of the sporulation-deficient mutants fail to modify the SPB, this SPB change might be indispensable for sporulation (Hirata and Shimoda, 1994). We have recently identified a novel coiled-coil protein, named Spo15, that is associated with the SPB and is essential for its modification (Ikemoto *et al.*, 2000).

After the SPB modification, membrane vesicles are gathered and fuse to generate forespore membranes near the modified SPB (Hirata and Tanaka, 1982; Tanaka and Hirata,

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Table 1. Strains

Strain	Mating type	Genotype	Source
TN29	h^{90}	*leu1-32 ura4-D18*	Ikemoto *et al.* (2000)
TN8	h^{90}	*leu1-32*	Nakamura *et al.* (2001)
TN52	h^{90}	*ade6-M210*	This work
MKW5	h^{90}		This work
B221	h^{90}	*spo14-B221 ade6-M210*	Bresch *et al.* (1968)
MK14	h^{90}	*spo14-B221*	This work
MK14L	h^{90}	*spo14-B221 leu1-32*	This work
MK05	h^{90}/h^{90}	*ade6-M210/ade6-M216*	This work
MK06	h^{90}/h^{90}	*spo14-B221/spo14-B221 ade6-M210/ade6-M216*	This work
MK07	h^{90}/h^{90}	*ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*	This work
MKD14	h^{90}	*ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 spo14$^+$/spo14::ura4$^+$*	This work
RM14	h^{90}	*spo14::ura4$^+$ ade6-M210 ura4-D18 leu1≪ spo14$^+$*	This work
RM14-7	h^{90}	*spo14::ura4$^+$ ade6-M216 ura4-D18 leu1≪ spo14-7*	This work
MK14H	h^{90}	*spo14::ura4$^+$ ade6-M210 leu1-32 ura4-D18* +pAL(spo14-HA)	This work
TN196	h^{90}	*spo14-B221 spo20-KC104 ade6-M210*	This work
TN226	h^{90}	*rer1-GFP≪ura4$^+$ ura4-D18*	This work
TN230	h^{90}	*rer1-GFP≪ura4$^+$ leu1-32 ura4-D18*	This work

1982). The sporulation-specific gene product Spo3 is a potential membrane protein and localizes to the forespore membrane (Nakamura *et al.*, 2001). The assembly of this membrane is defective in the *spo3* null mutant. One of the *spo3* alleles, *spo3-KC51*, is dose-dependently suppressed by *psy1$^+$*, which encodes a protein similar to mammalian syntaxin-1A, a component of the plasma membrane docking/fusion complex. In fact, Psy1 localizes to the plasma membrane during vegetative growth. The *psy1$^+$* gene is essential for vegetative growth, and its transcription is further enhanced during sporulation. Interestingly, Psy1 disappeared from the plasma membrane of mother cells immediately after the first meiotic division and relocalized to the nascent forespore membrane. These results support the idea that the forespore membrane is assembled by the fusion of membrane vesicles assisted by a syntaxin-like protein Psy1 (Nakamura *et al.*, 2001).

The origin of these vesicles for the extension of the forespore membrane remains to be elucidated. One plausible mechanism is that the forespore membranes are assembled by the fusion of vesicles from the endoplasmic reticulum (ER) and/or the Golgi apparatus. Transport of membrane vesicles is carried out by a set of *SEC* gene products. The yeast secretory pathway was elucidated by analyzing a number of temperature-sensitive mutants that are defective in the regulatory or constitutive transport machinery (Novick *et al.*, 1980). Some late-acting *SEC* genes have been reported to be necessary for sporulation in both *Saccharomyces cerevisiae* and *S. pombe* (Neiman, 1998; Nakase *et al.*, 2001).

In this study, we have characterized the *spo14$^+$* gene product and analyzed phenotypes of *spo14* mutants. Spo14 is identical to Stl1 (d'Enfert *et al.*, 1992), which was supposed to be structurally and functionally related to the *S. cerevisiae* Sec12. Sec12 is a type II membrane protein, which regulates vesicle transport from the ER to the Golgi apparatus (Novick *et al.*, 1980; Nakano *et al.*, 1988; Kaiser and Schekman, 1990; d'Enfert *et al.*, 1991, Rexach and Schekman, 1991). This protein is known as a GEF (GTP-GDP exchange factor) for a small GTPase, Sar1 (Barlowe and Schekman, 1991), which plays an essential part in the formation of transport vesicles at the ER (Nakano and Muramatsu, 1989; d'Enfert *et al.*, 1991). Our analyses reported here support the notion that Spo14 is an *S. pombe* homologue of the budding yeast Sec12 that is essential for vesicle budding from ER. Furthermore, we examined defects in the forespore membrane assembly in *spo14-B221* mutants in detail to elucidate the significance of the proteins in the secretion pathway in sporulation.

MATERIALS AND METHODS

Yeast Strains, Media, and Transformation

The *S. pombe* strains used in this study are listed in Table 1. The media used in this study have been previously described (Egel and Egel-Mitani, 1974; Gutz *et al.*, 1974; Moreno *et al.*, 1990). *S. pombe* cells were grown at 30°C and sporulated at 28°C unless otherwise stated. For examination of the sporulation defects in the *spo14-B221* mutant, sporulation was induced at 23°C.

Cloning of spo14$^+$

The *spo14-B221* mutant (MK14L) was transformed with an *S. pombe* genomic library containing partial *Sau*3AI DNA fragments constructed in a multicopy plasmid, pDB248′ (Beach and Nurse, 1981). Approximately 10^5 independent Leu$^+$ transformants were obtained. These transformants were allowed to sporulate on selective SSA plates, and they were then treated with 30% ethanol for 30 min to kill nonsporulating vegetative cells (Gutz *et al.*, 1974). Cells were then spread again on SSA sporulation plates, which were then exposed to iodine vapor (Gutz *et al.*, 1974). Iodine-positive (brown) colonies were removed and inspected for recovery of sporulation. Plasmid DNA was transferred from some of the Leu$^+$ Spo$^+$ colonies to an *Escherichia coli* strain (DH5α). Two isolated plasmids, designated pDB(spo14)1 and pDB(spo14)2, contained ~8- and 3-kb DNA inserts, respectively. Purified plasmids completely complement the *spo14-B221* mutant. Sequencing of pDB(spo14)2 revealed that the insert contained one open reading frame (ORF) that was identical to *stl1$^+$* (d'Enfert *et al.*, 1992). Subcloning of pDB(spo14)1 confirmed that this ORF is responsible for the complementation of *spo14-B221* (Figure 1A). This ORF represents the *spo14$^+$* gene itself, but not the multicopy suppressor, as described below.

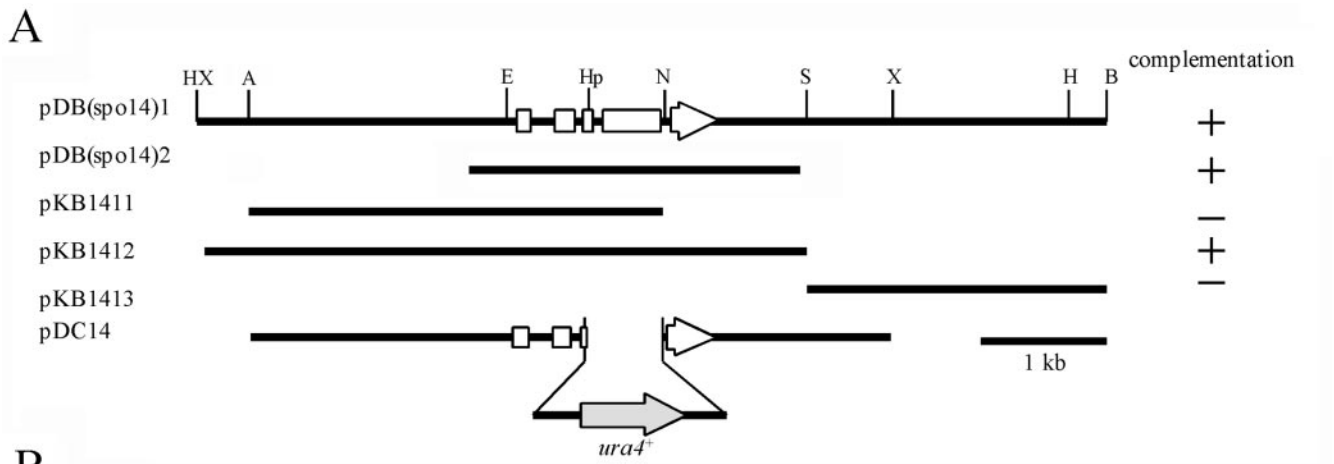

Figure 1. Cloning, disruption, and the nucleotide sequence of the *spo14*$^+$ gene. (A) Restriction map, subcloning, and disruption of the *spo14*$^+$ gene. Plasmids pDB(spo14)1 and pDB(spo14)2 were independently isolated. The white arrow indicates the region and direction of the *spo14*$^+$ ORF. Restriction enzyme sites: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; N, *Nsi*I; S, *Sac*I; X, *Xba*I. (B) Nucleotide sequence of *spo14*$^+$ and its predicted amino acid sequence. Putative introns are underlined. A region indicated by bold letters is a hydrophobic stretch of 20 amino acid residues.

Disruption of the spo14$^+$ *Gene*

The plasmids used for gene disruption were constructed as follows. A 6.0-kb of *Apa*I-*Xba*I fragment containing the *spo14*$^+$ ORF was cloned into the corresponding site of pBluescript II-KS$^+$ (Stratagene, La Jolla, CA). A 0.7-kb *Hpa*I-*Nsi*I fragment of the resulting plasmid was replaced by *ura4*$^+$, yielding pDC14. (Figure 1A). The linear *Apa*I-*Xba*I fragment containing the interrupted *spo14* allele was used to transform the strain MK07. Disruptions were confirmed by genomic Southern hybridization.

Plasmid Construction

Plasmids used in this work are listed in Table 2. Plasmid pKB282 was constructed as follows. The *Nde*I-*Nde*I region of pDblet (Brun *et al.*, 1995) containing the *ura4*$^+$ gene was eliminated, filled in, and then ligated with *Bgl*II linker, yielding pDblet-dash. pDblet-dash was digested with *Bgl*II and ligated with a 2.9-kb *ade6*$^+$ fragment, yielding pKB282. To examine whether the overproduction of the *spo14-B221* gene can resolve the sporulation defect of the *spo14-B221*, the *spo14-B221* allele was cloned directly from the *spo14-B221* genomic DNA by PCR using the primers 5′-CCCGGGCCC(*Apa*I)TTCATAATGGCTATAG-3′ and 5′-CCCGAGCTC(*Sac*I)TTTCATTCAT AGTTATG-3′. The resulting fragment was digested with *Apa*I and *Sac*I, and cloned into pAL-KS (Tanaka *et al.*, 2000) to create pAL(spo14-B221). Plasmid pBR(leu1) was constructed by inserting *leu1*$^+$ gene into the *Pvu*II site of pBR322. Plasmid pGEX-KG(*Not*I) was constructed by inserting *Not*I linker into the *Hin*dIII site of pGEX-KG (Guan and Dixon, 1991). Plasmid pAL (spo14-HA) was

Table 2. Plasmids

Plasmid	Characteristics	Source of reference
pDB248′	2μm origin, *LEU2*-based vector	Beach and Nurse (1981)
pAL-KS	*ars1, LEU2*-based vector	Tanaka *et al.* (2000)
pAU-SK	*ars1, URA3*-based vector	Tanaka *et al.* (2000)
pREP1	*ars1, LEU2*-based vector carrying a thiamine-repressible *nmt1* promoter	Maundrell (1993)
pREP41	*ars1, LEU2*-based vector carrying a thiamine-repressible *nmt41* promoter	Maundrell (1993)
pREP81	*ars1, LEU2*-based vector carrying a thiamine-repressible *nmt81* promoter	Maundrell (1993)
pTN143	GFP and *nmt1* terminator in pAL-KS	Ikemoto *et al.* (2000)
pSLF372	3XHA and *nmt81* promoter	Forsburg and Sherman (1997)
pTN280	3XHA and *nmt81* promoter	This work
pTN144	3XHA and *nmt1* terminator in pAL-KS	This work
pTN253	3XHA and *nmt1* terminator in pAU-SK	This work
pTN281	GFP in pREP81	This work
pBR(leu1)	*leu1*$^+$ in pBR322	This work
pDblet	*ars2004*X2, *ura4*$^+$-based vector	Brun *et al.* (1995)
pKB282	*ade6*$^+$ in pDblet	This work
pIUGFP	GFP and *nmt1* terminator in *ura4*$^+$-based integration vector	This work
pDB(spo14)1	Genomic *spo14*$^+$ isolated from as *S. pombe* genomic library	This work
pDB(spo14)2	Genomic *spo14*$^+$ isolated from as *S. pombe* genomic library	This work
pAL(spo14)	*spo14*$^+$ in pAL-KS	This work
pDC(spo14)	for *spo14* disruption	This work
pREP41(spo14)	*spo14*$^+$ in pREP41	This work
pREP41(SEC12)	*SEC12* in pREP41	This work
pREP41(psr1)	*psr1*$^+$ in pREP41	This work
pAL(spo14-B221)	*spo14-B221* in pAL-KS	This work
pAL(spo14-HA)	*spo14*$^+$ in pTN144	This work
pAU(spo14-HA)	*spo14*$^+$ in pTN253	This work
pREP81(13g6-GFP)	13g6 in pTN281	This work
pREP1(gma12-HA)	gma12-HA in pREP1	T. Yoko-o
pREP81(GFP-psy1)	*GFP-psy1* in pREP81	Nakamura *et al.* (2001)
pAL(spo3-GFP)	*spo3*$^+$ in pTN143	Nakamura *et al.* (2001)
pKB282(rer1-GFP)	*rer1*-GFP in pKB282	This work
pIU(rer1-GFP)	*rer1*$^+$ in pIUGFP	This work

constructed as follows. A 1.8-kb *Not*I-*Sac*I fragment of pSLF372 (Forsburg and Sherman,1997), which contains the HA epitope and the *nmt1* terminator (Maundrell, 1993), was ligated into the corresponding sites of pAL-KS to create pTN144. The 5-kb *Apa*I-*Xba*I fragment of pDB(spo14)1 was inserted at the corresponding sites in pTN144, yielding pAL(spo14I-HA). Two oligonucleotides were used to amplify the C terminal region of the *spo14*$^+$ gene by PCR using 5′-CCCGTCGAC(*Sal*I)AATGGCTGAACTCCAC-3′ and 5′-ATTTGCGGCCGC(*Not*I)AAAGGTCATAGTTTT-3′ as primers. The PCR product was digested with *Nsi*I and *Not*I and then ligated into the corresponding site of pAL(spo14I-HA), yielding pAL(spo14-HA). Plasmid pREP81(13g6-GFP) was constructed as described by Brazer *et al.* (2000). Plasmid pKB282(rer1-GFP) was constructed through two steps. The *rer1*$^+$ gene was amplified by PCR using 5′-CCCGTCGAC(*Sal*I)AATGCTTGCAATCTTGTC-3′ and 5′-CCC-GCGGCCGC(*Not*I)AGTGTGAGCCAAATTTTTTCTTACCG-3′ as primers. The resulting PCR product was digested with *Sal*I and *Not*I and ligated into the corresponding site of pTN143, yielding pAL-(rer1-GFP). As the second step, pAL(rer1-GFP)was digested with *Apa*I and *Sac*I and cloned into the corresponding site of pKB282 to create pKB282(rer1-GFP). Plasmid pIU(rer1-GFP) was constructed as follows. Two oligonucleotides were used to amplify the *rer1*$^+$ gene by PCR using 5′-CCCGTCGAC(*Sal*I)CATGGAATTCAT-TCAGCGTC-3′ and 5′-CCCGCGGCCGC(*Not*I)AGTGTGAGCCAA-ATTTTTTCTTACCG-3′ as primers. The resulting PCR product was digested with *Sal*I and *Not*I and ligated into the *Xho*I and *Not*I sites of pIUGFP, yielding pIU(rer1-GFP). The pIU(rer1-GFP) plasmid was linearized by restricting it with *Sph*I near the center of the *rer1*$^+$ sequence and introduced into the strain (TN29).

Generation of Anti-Spo14 Antibody

A GST-tagged protein of Spo14 produced in *E. coli* was used for the polyclonal antibodies. GST-Spo14 was obtained as follows. A 1.2-kb DNA fragment carrying the entire *spo14* coding region was amplified by PCR with two oligonucleotides, 5′-CCCGTCGAC(*Sal*I)AAT-GGCTGAACTCCAC-3′ and 5′-ATTTGCGGCCGC(*Not*I)AAAGGT-CATAGTTT-3′. The amplified DNA was digested with *Sal*I and *Not*I and then inserted into the same sites of the GST-tag expression vector pGEX-KG(Not) to make pGEX(spo14). The pGEX(spo14) was digested with *Sph*I and *Not*I to eliminate the C-terminal region of Spo14, filled in, and self-ligated, yielding pGEX(spo14S), and the pGEX(spo14S) was transformed into *E. coli* BL21. The fusion protein was purified from SDS gels and used to immunize rabbit. Rabbit sera thus produced a 45-kDa protein in crude yeast extracts on an immunoblot (Figure 2A). For affinity purification, the sera were loaded over an AffiGel-15 (Bio-Rad, Hercules, CA) column containing the purified denatured GST-tagged Spo14 and eluted with 4.5 M $MgCl_2$. Eluates were immediately dialyzed against PBS containing 30% glycerol and used for Western blot analysis.

Immunofluorescence Microscopy

For cell fixation, we followed the method of Hagan and Hyams (1988) and used glutaraldehyde and paraformaldehyde. The Spo14-HA and Gma12-HA were visualized by indirect immunofluorescence microscopy using rat anti-HA antibody 3F10 (Boehringer Mannheim, Mannheim, Germany) and Alexa 488– or Alexa 594–conjugated goat anti-rat IgG (Molecular Probes, Eugene, OR). The

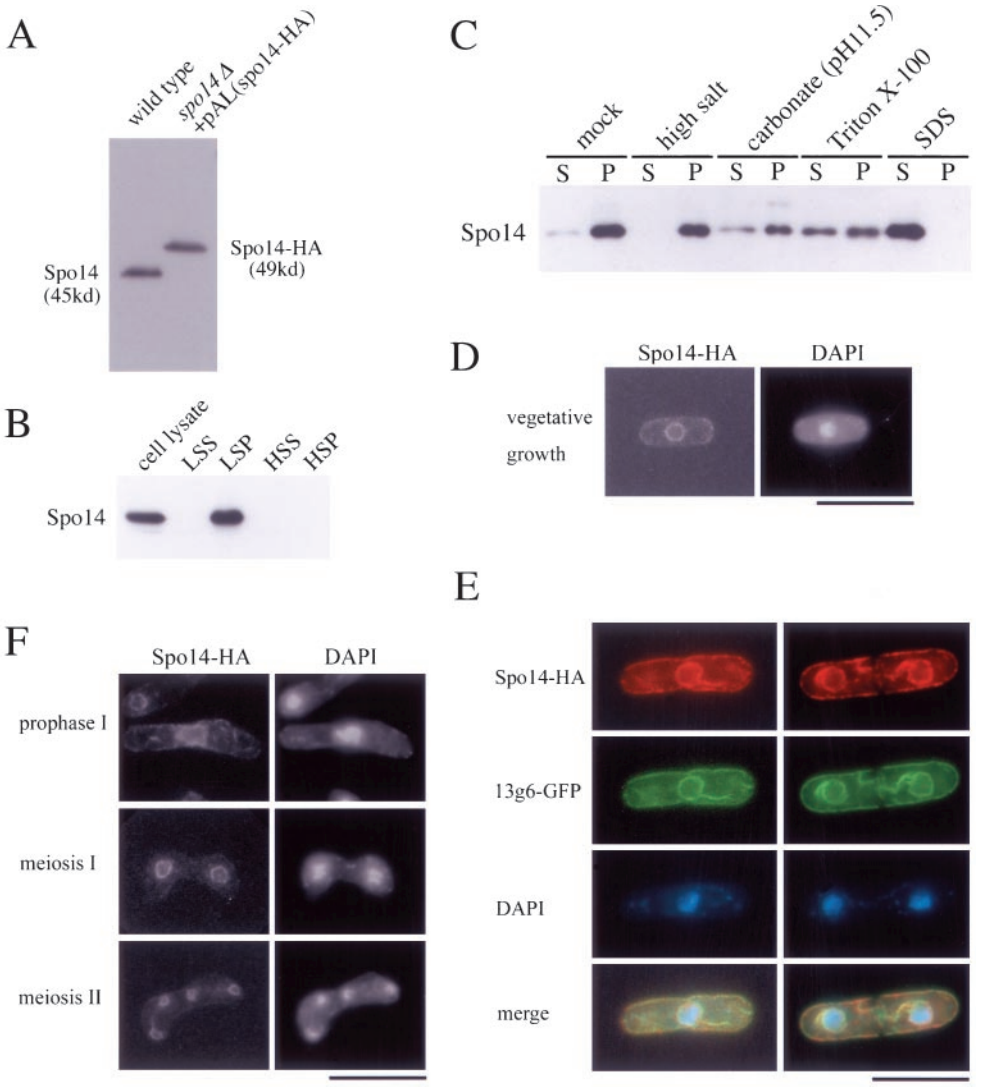

Figure 2. Localization of Spo14 to the ER membrane. (A) Western blot analysis of *S. pombe* cell extracts using anti-Spo14 antibody. MKW5 (wild-type) and MK14H (*spo14*Δ+ pAL(spo14-HA)) cells were grown in liquid complete medium (YE). Protein extracts were subjected to immunoblot analysis with the anti-Spo14 antibody. (B) Subcellular fractionation of membranes. MKW5 cells were spheroplasted and homogenated. The cell lysates was subjected to differential centrifugation to separate LSP, HSP, and HSS fractions as described in Elagoz *et al.* (1999). Each fraction was resolved on SDS-PAGE and subjected to immunoblot analysis using anti-Spo14 antibody. (C) The LSP fraction was extracted with lysis buffer (mock) or the same buffer containing either 0.5 M NaCl (high salt), 0.1 M sodium carbonate (pH 11.5), 1.5% Triton X-100, or 0.1% SDS. After extraction, mixtures were fractionated into the supernatant (S) and the pellet (P) by centrifugation (100,000 × *g*, 1 h). Each fraction was analyzed by Western blotting using anti-Spo14 antibody. (D) Localization of Spo14-HA in vegetative cells. Strain MK14H was cultured in MM medium and processed for immunofluorescence microscopy with anti-HA mAb (3F10) followed by Alexa 488-conjugated anti-rat IgG antibody. Bar, 10 μm. (E) Colocalization of Spo14-HA with an ER marker protein, 13g6. A wild-type strain TN29 carrying pAU(Spo14-HA) and pREP81(13g6-GFP) was grown to midlog phase in MM at 28°C. After fixation, cells were stained with anti-HA antibody (3F10) followed by Alexa 594-conjugated anti-rat IgG antibody and DAPI. Bar, 10 μm. (F) Localization of Spo14 during meiosis. Strain MK14H carrying pAL(spo14-HA) was cultured in SSL−N to induce meiosis. Spo14-HA was visualized as described in Figure 2D. Bar, 10 μm.

SPB was visualized by indirect immunofluorescence microscopy using rabbit anti-Sad1 antibody and Alexa 546-conjugated goat anti-rabbit IgG (Molecular Probes). For microtubule staining, TAT-1 anti-α-tubulin antibody (Woods *et al.*, 1989) and Cy3-conjugated secondary antibody (Sigma Chemical Co., St. Louis, MO) were used. To visualize the nuclear chromatin region, we stained the cells with 4′,6-diamidino-2-phenylindole (DAPI) at 1 μg/ml. Stained cells were observed under a fluorescence microscope (model BX50; Olympus, Tokyo, Japan) equipped with a charge-coupled device (CCD) camera (Cool-SNAP; Roper Scientific, San Diego, CA).

RT-PCR

Total RNA was prepared from *S. pombe* cultures (Jensen *et al.*, 1983). Reverse transcription (RT)-PCR was performed with a commercial kit (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden), and the treatment of total RNA with DNase I before RT-PCR was carried out according to the supplier's instructions. The forward and reverse primers for PCR were 145s 5′-GCTGGAGCAGATTGCAGC-3′ and 145as 5′-ATTTGCGGCCGCAAAGGTCATAGTTTT-3′.

In Vitro Mutagenesis of spo14 by PCR

To screen for conditionally lethal alleles, the whole *spo14*$^+$ ORF was randomly mutagenized by the error-prone PCR method (Leung *et al.*, 1989). The forward and reverse primers for PCR were 5′-CCCAGTGGAATTC(*Eco*RI)TTGTAAGTGTAC-3′ and 5′-CCC-GAGCTC(*Sac*I)TTTCATTCATAGTTATG-3′, respectively. The amplified DNA fragment contained the promoter and terminator regions, besides the coding region, of the *spo14* gene. PCR was carried out in a reaction mixture composed of mutagenesis buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1 mM $MgCl_2$, 0.01% Triton X-100, and 0.1 mM $MnCl_2$) and primers, recombinant *Taq* DNA polymerase (Toyobo, Osaka, Japan) and dNTPs (2 mM each). Plasmid pAL-

(spo14) was included in the reaction mixture as a template. The amplified fragment was digested with *Eco*RI and *Sac*I and cloned into pBR (leu1) vector. The resulting library was digested at the *Nru*I site within the *leu1*$^+$ gene and then integrated at the *leu1* locus of the *spo14* disruptant (MKD14). The transformant colonies on SSA plates were treated with ethanol to kill nonsporulating vegetative cells and then spread on minimal medium. Candidates were replica-plated onto minimal plates containing phloxin B, and they were then incubated at either 20 or 37°C. Two novel *spo14* mutants that showed cold-sensitive growth were further characterized.

Nucleotide Sequence Analysis of the spo14 Mutant Alleles

The entire *spo14* ORF and the promoter region were amplified by PCR using genomic DNA from the *spo14-B221*, *spo14-6*, or *spo14-7* as a template and then cloned into pAL-KS. The nucleotide sequences of six clones derived from each independent PCR amplification were determined in their entirety.

Nucleotide Sequence Accession Number

The sequence data for *spo14*$^+$ are available from EMBL/GenBank/DDBJ under accession no. AB036755.

RESULTS

The Spo14-B221 Mutant Shows Defects in Ascospore Formation but Not in Vegetative Growth or Meiotic Nuclear Division

As we previously reported, *spo14-B221* mutants exhibit cold sensitivity in ascospore formation (Kishida and Shimoda, 1986; Kishida *et al.*, 1990; Hirata and Shimoda, 1992). To investigate meiosis and sporulation in the *spo14-B221* mutant in more detail, we monitored meiotic nuclear divisions and ascus formation in homozygous diploid strains MK05 (*spo14*$^+$/*spo14*$^+$) and MK06 (*spo14-B221/spo14-B221*). At both 23 and 30°C, first and second meiotic divisions in the *spo14-B221* mutant proceeded with kinetics similar to those in the isogenic wild-type strain. However, virtually no asci were produced in the *spo14-B221* strain at 23°C. These results suggest that the *spo14-B221* mutant is able to complete meiosis, but that it is defective in ascospore formation.

Next, we observed effects of the *spo14-B221* mutation on properties of vegetative growth. The growth rate of the *spo14-B221* mutant was not significantly different from those of wild-type cells. Cell size and morphology of the mutant were also normal. We thus concluded that the *spo14-B221* mutant proceeds through normal mitotic and meiotic divisions but shows defects in ascospore formation.

The spo14$^+$ *Gene Encodes a Functional Homologue of Budding Yeast Sec12*

The *spo14*$^+$gene was cloned by means of its ability to complement the sporulation defect of the *spo14-B221* mutant (MATERIALS AND METHODS). This gene is identical to the previously characterized *stl1*$^+$ gene, which is a putative homologue of the *S. cerevisiae SEC12* gene (d'Enfert *et al.*, 1992). The comparison of the genomic sequence (this study) with the corresponding cDNA sequence (d'Enfert *et al.*, 1992) shows that the predicted *spo14*$^+$ gene is split by five introns (Figure 1B). Interestingly, the first intron is located in the 5′ untranslated region of the gene (Figure 1B). We refer to this gene as *spo14*$^+$ hereafter in this article.

To determine the consequences of complete loss of *spo14*$^+$ function, we disrupted the *spo14*$^+$ gene using a plasmid, pDC(spo14), constructed by deleting ~50% of the ORF and replacing this with the *ura4*$^+$ gene (Figure 1A). Tetrad analysis indicated that every ascus contained two viable and two inviable spores and that all viable spores were Ura$^-$. Microscopic observation of nonviable progenies showed that spores germinated but ceased growth after a few divisions. This demonstrates that *spo14*$^+$ is essential for vegetative growth and viability. A *spo14-B221/spo14*Δ diploid strain could not form spores, indicating that the cloned gene is *spo14*$^+$ itself.

Generally, the *S. pombe* genes responsible for mating, meiosis and sporulation are transcribed under conditions of nutritional starvation (Yamamoto *et al.*, 1997; Horie *et al.*, 1998; Abe and Shimoda, 2000). Northern analysis revealed that *spo14*$^+$ was transcribed during growth and was not further enhanced after the shift to a nitrogen-free medium (Figure 6C). In summary, Spo14 functions not only in sporulation but also in vegetative growth.

Identification of Spo14 Proteins

To identify and characterize the *spo14*$^+$ gene product, we generated a rabbit polyclonal antiserum raised against a GST-fused Spo14 (see MATERIALS AND METHODS). This antiserum was used to detect Spo14 in crude *S. pombe* cell extracts by immunoblotting. The antibody recognized a single band with an apparent molecular mass of 45 kDa, which was in accord with that calculated from the nucleotide sequence (Figure 2A). In *spo14*Δ cells expressing HA-tagged Spo14, the band was shifted upward due to the tagging peptide (~49 kDa; Figure 2A). Therefore, we concluded that the antibody specifically recognized the *spo14*$^+$ gene product. *S. cerevisiae* Sec12 is known to be glycosylated (Nakano *et al.*, 1988). The apparent molecular mass was not decreased after treatment with endo H glycosidase, suggesting that Spo14 is not strikingly glycosylated.

Spo14 Localizes to the ER

To elucidate the localization of Spo14, we first conducted subcellular fractionation experiments. Cells were converted to spheroplasts by lyticase treatment, homogenized, and subjected to differential centrifugation. In Western blotting using anti-Spo14 antibody (Figure 2B), Spo14 was almost exclusively present in a low-speed pellet (LSP) fraction that contained ER and vacuoles. To test whether Spo14 was soluble in a membrane-enclosed compartment or was an integral membrane protein, the LSP fraction was treated with high concentrations of salt solution, an alkaline solution, and detergents. Figure 2C shows that Spo14 remained sedimentable after treatment with a high concentration of salts and alkali but was partially solubilized by detergents. These facts suggested a firm association of Spo14 with the lipid bilayer.

To analyze further the localization of Spo14, we performed immunofluorescence microscopy. Because the polyclonal anti-Spo14 antibody mentioned above could not detect Spo14 protein by indirect immunofluorescence, we used the *spo14*Δ strain expressing Spo14-HA, which could be detected by an-

ti-HA antibody. The *spo14*Δ cells containing either single or multiple copy Spo14-HA grew and sporulated normally. In vegetative cells, the Spo14-HA fluorescence gave a ring-like staining pattern surrounding the nucleus, and there was also staining in the cell periphery (Figure 2D). Essentially identical data were obtained with strains harboring a single copy of the spo14-HA fusion allele integrated chromosomally. Typical ER-resident proteins show similar staining images. The protein tentatively named 13g6 has been demonstrated to be localized to the ER (Brazer *et al.*, 2000). Thus, Spo14-HA was coexpressed with 13g6-GFP. As shown in Figure 2E, both proteins were clearly colocalized, demonstrating that Spo14 is associated with the ER.

We also investigated Spo14 localization during meiosis and sporulation. The Spo14-HA was localized around nuclei during the sporulation process. Interestingly, signals at the cell periphery disappeared (Figure 2F). This observation suggests that intracellular membranes including the ER and Golgi apparatus possibly undergo dynamic alterations in this cell reforming process.

Isolation of Conditional Lethal Spo14 Mutants

S. cerevisiae Sec12 plays an essential part in protein secretion. To determine whether Spo14 is involved in this process, we attempted to isolate conditional lethal mutants by random PCR mutagenesis (see MATERIALS AND METHODS). We isolated two low temperature-sensitive mutants, *spo14-6* and *spo14-7,* both of which grew normally at 28 and 37°C but poorly at 20°C. These mutant alleles were amplified by PCR and sequenced. The *spo14-6* mutation caused a single nucleotide change (from T to C) at the 101st nucleotide from the initiation codon, resulting in valine to alanine replacement at the amino acid position 34. The *spo14-7*allele contained a single nucleotide change (from T to C) at the 1201st nucleotide that caused a change from leucine to histidine at amino acid position 299. Both mutation sites were located in the cytoplasmic region of Spo14 protein.

Because *SAR1* suppresses the temperature sensitivity of *sec12* mutants in *S. cerevisiae* (Nakano and Muramatsu, 1989), it is possible that the corresponding homologues of *S. pombe* may interact with each other. In fission yeast, an SAR*1*-like gene, once designated *sar1*$^+$ (d'Enfert *et al.*, 1992), has been isolated by the suppression of temperature-sensitive growth of the budding yeast *sec12* mutant. Because the gene symbol *sar1*$^+$ has already been used (Wang *et al.*, 1991), we propose a new nomenclature, *psr1*$^+$ (for *pombe* S*a*r1). To examine whether overproduction of Psr1 suppresses the *spo14* mutation in *S. pombe*, we introduced a multicopy plasmid harboring *psr1*$^+$ into *spo14-6* and *spo14-7* strains. Overexpression of *psr1*$^+$ complemented the cold sensitivity of both the *spo14-6* and the *spo14-7* mutants.

Spo14 Functions in the Vesicle Transport from the ER to the Golgi Apparatus in Vegetative Growth

To determine whether protein transport was defective in the conditional lethal *spo14-7* mutant, we determined the terminal phenotypes of the mutant after incubation at 20°C for 4 h. Prominent ER-like membrane structures were accumulated in the cytoplasm of *spo14-7* mutant cells. Furthermore, invagination of plasma membranes and abnormal nuclear structures were often observed in the mutant cells (Figure 3, B, C, E, and F). Similar results were obtained when *spo14-6* mutant was incubated at the restrictive temperature. In contrast, no abnormal membrane structure was accumulated in wild-type cells (Figure 3, A and D) or *spo14-B221* cells.

To confirm that Spo14 regulates the ER-Golgi protein transport, we observed the localization of *S. pombe* Rer1 protein in the *spo14* mutant. *S. cerevisiae* Rer1 is a membrane protein present in the Golgi apparatus, and it is required for the retrieval of a variety of ER membrane proteins (Nishikawa and Nakano 1993; Boehm *et al.* 1994, 1997; Sato *et al.*, 1995, 1997). Rer1 directly interacts with the transmembrane domain of Sec12, which contains a retrieval signal (Sato *et al.*, 1996, 2001). The *S. cerevisiae* Rer1 protein fused to GFP rapidly shuttles between the Golgi apparatus and the ER. In the mutant, where membrane traffic from the ER to the Golgi apparatus is prevented, Rer1 localization is restricted to the ER (Sato *et al.*, 2001). Rer1 is conserved evolutionarily among eukaryotes, and in fact, the *S. pombe* genome also contains a single copy of the homologous gene (Sato *et al.*, 1999). We named this gene *rer1*$^+$. To verify that Rer1 resides preferentially in the Golgi apparatus, we examined the subcellular localization of Rer1-GFP in the strain TN230 carrying a single chromosomal copy of the *rer1*$^+$ gene tagged with GFP and driven by the authentic promoter. Previous studies have shown that ER and vacuolar membranes distribute mainly in the low-speed pellet (LSP) fraction, whereas Golgi membranes are recovered in both LSP and high-speed pellet (HSP) fractions (Nakano *et al.*, 1988; Gaynor *et a*l., 1994). Subcellular fractionation experiments revealed that Rer1 was present in both LSP and HSP fractions (Figure 4A). Rer1-GFP was visualized as numerous dots scattered in the cytoplasm. Localization of Rer1 in wild-type cells was compared with that of Gma12, Golgi-associated protein (Chappell *et al.*, 1994). A fluorescent microphotograph indicates that Rer1-GFP and Gma12-HA are largely colocalized in the cytoplasm (Figure 4B). These observations strongly suggest that *S. pombe* Rer1 may also localize to the Golgi apparatus. We noticed, however, that fluorescent signals of Rer1-GFP and Gma12-HA were not completely overlapped. Perhaps, this is due to the differences in their localization within the Golgi apparatus. In fact, *S. cerevisiae* Rer1 is mostly present in early stages of Golgi apparatus formation, its localization is slightly different from that of Kex2, a trans-Golgi protein (Sato *et al.*, 1995).

Next, we observed the localization of Rer1-GFP in *spo14* mutants. In wild-type and *spo14-B221* cells, Rer1-GFP signals showed punctate distribution, thus suggesting a Golgi apparatus localization, at either 30 or 20°C. In the cold-sensitive *spo14-7* mutant, however, the Rer1-GFP signal showed an ER-like pattern at a restrictive temperature (20°C; Figure 4C). Similar results were obtained with the *spo14-6* cold-sensitive mutant. Brefeldin A is known to stimulate the membrane recycling from the Golgi apparatus to the ER in many organisms, including fission yeast (Pelham, 1991; Klausner *et al.*, 1992; Turi *et al.*, 1994; Brazer *et al.*, 2000). The treatment with this drug altered the Rer1-GFP signal from the punctate (Golgi) pattern to the ER pattern as observed in *spo14-6* and *spo14-7* (Figure 4D). In conclusion, Spo14 is involved in the membrane traffic from the ER to the Golgi apparatus, like *S. cerevisiae* Sec12.

In budding yeast, secretion of the periplasmic proteins such as invertase and acid phosphatase is blocked in condi-

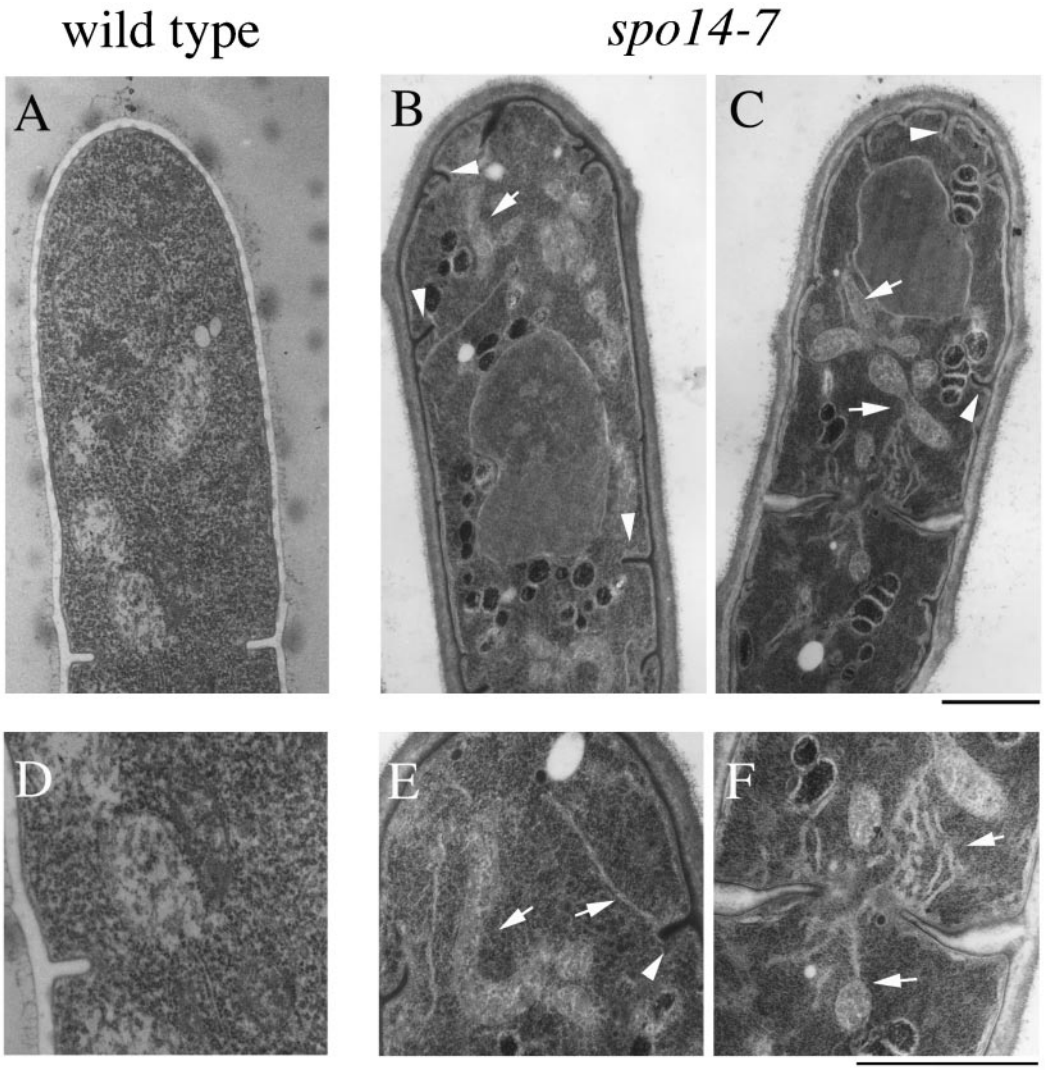

Figure 3. Fine structures of the conditional *spo14-7* mutants at a restrictive temperature. RM14 and RM14-7 were grown to a midlog phase at 30°C and then shifted to the restrictive temperature (20°C). After incubation for 4 h, cells were fixed for electron microscopy. Arrowheads and arrows represent invagination of plasma membranes and the ER-like structures, respectively. A, RM14; B and C, RM14-7. D, E, and F, higher magnified images of A, B, and C, respectively. Bars, 1 μm.

tional *sec* mutants at restrictive temperatures (Novick *et al.*, 1980, 1981; Ferro-Novick *et al.*, 1984). However, the secretion of invertase and acid phosphatase is not blocked in *S. pombe* conditional *spo14* mutants, although the glycosylation of the proteins was defective. Recently, we showed that the secretion of invertase and acid phosphatase was not affected in another secretion mutant, *spo20-KC104*, at the restrictive temperature, whereas the Golgi apparatus is accumulated (Nakase *et al.*, 2001). The mechanism of the secretion in *S. pombe* might be partially different from that in *S. cerevisiae*.

Sporulation Deficiency of spo14-B221 Mutants is Due to Reduction in the Abundance of spo14 mRNA

Next, we examined the role of Spo14 in sporulation. To determine whether *S. cerevisiae SEC12* complements the sporulation defect of the *spo14-B221* mutant, we introduced *SEC12* driven by the *nmt1* promoter into the *S. pombe spo14-B221* mutant. This expression plasmid, pREP41(SEC12), rescued the *spo*$^-$ phenotype (Figure 5A). The degree of suppression of sporulation deficiency by the expression of *SEC12* was comparable to that achieved by the expression of *spo14*$^+$ (Figure 5B), and most spores were viable. Thus, we conclude that *S. pombe* Spo14 plays a similar role to that of *S. cerevisiae* Sec12 not only in vegetative growth but also in sporulation.

As mentioned above, *S. cerevisiae SAR1* suppresses the phenotype of temperature-sensitive *sec12* mutants. In addition, overexpression of *psr1*$^+$ suppresses the temperature sensitivity of budding yeast *sec12* (d'Enfert *et al.*, 1992). To examine whether overproduction of Psr1 suppresses the

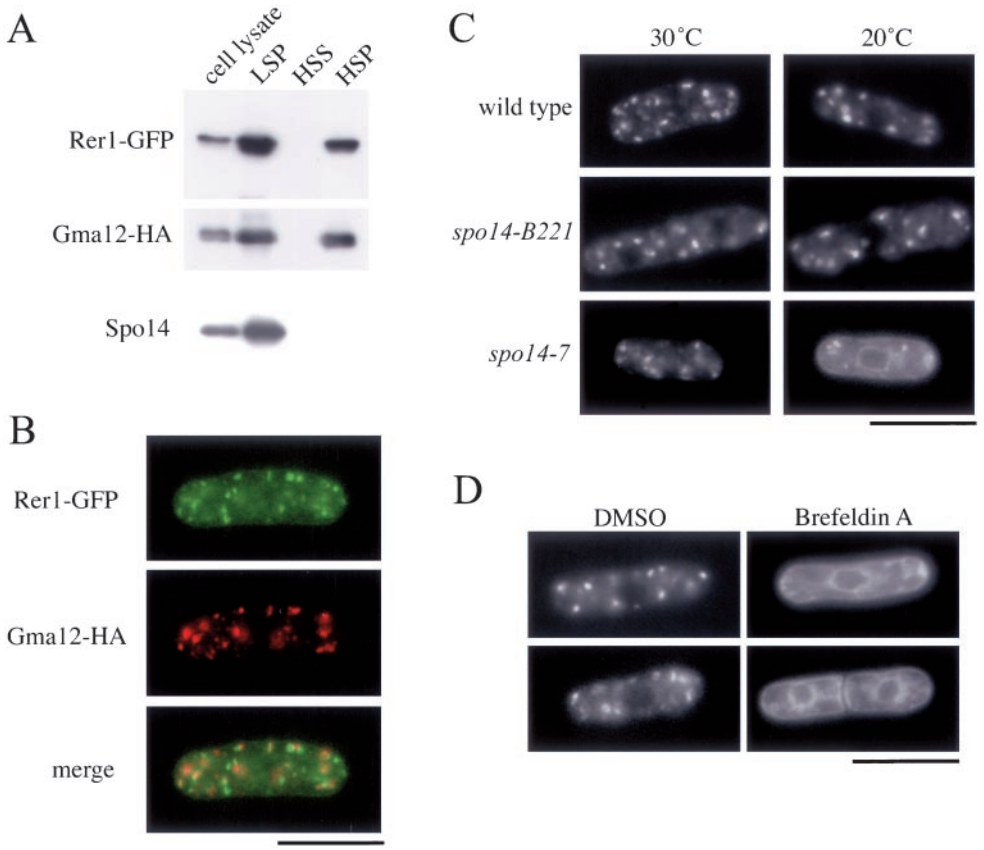

Figure 4. Effect of the *spo14-7* mutation on the ER/Golgi shuttling protein, Rer1. (A) Subcellular fractionation of Rer1-GFP. Strain TN230 transformed with pREP1(gma12-HA) was cultured in MM supplemented with thiamine (20 μM) at 28°C. Cells were converted to spheroplasts, homogenized, and subjected to differential centrifugation to fractionate into LSP, HSP, and HSS. Each fraction was resolved by SDS-PAGE and subjected to immunoblot analysis using either anti-GFP, anti-HA, or anti-Spo14 antibody to detect Rer1-GFP, Gma12-HA, and Spo14, respectively. (B) Double-immunofluorescence staining of Rer1 and Gma12. Strain TN230 transformed with pREP1(gma12-HA) was cultured in MM minimal medium supplemented with thiamine (20 μM) at 28°C. Cells were then stained with anti-HA antibody followed by Alexa 594–conjugated anti-rat IgG antibody and DAPI. Bar, 10 μm. (C) ER-to-Golgi transport was blocked in *spo14-7*. TN52 (wild-type), B221 (*spo14-B221*), and RM14-7 (*spo14-7*) cells were transformed with pKB282(rer1-GFP). These strains were grown in liquid medium (MM) at 20°C for 3 h. Localization of Rer1-GFP was observed under a fluorescence microscope. Bar, 10 μm. (D) Effect of Brefeldin A on the localization of Rer1-GFP. Strain TN226 (wild-type) was incubated in MM medium to log phase. Brefeldin A dissolved in dimethyl sulfoxide (DMSO) was added to the culture medium to a final concentration of 100 μg/ml. After incubation for 2 h, Rer1-GFP was observed under a fluorescence microscope. Bar, 10 μm.

sporulation defect of the *spo14-B221* mutation in *S. pombe*, we introduced a multicopy plasmid harboring *psr1*$^+$ into *spo14-B221* strains. The transformants recovered the sporulation-deficient phenotype (Figure 5, C and D), and the produced spores were viable. These results strongly suggest that Spo14 regulates Psr1 in a manner analogous to that of *S. cerevisiae* Sec12 and Sar1.

As described above, *spo14*$^+$ is an essential gene, whereas the *spo14-B221* mutant shows a sporulation-specific phenotype. Nucleotide sequence analysis demonstrated that *spo14-B221* contained a single nucleotide change (from T to A) in the fifth intron (Figure 6A). The branch point consensus sequence CURAY was mutated to CURA*A* in *spo14-B221*. To examine whether splicing efficiency of the fifth intron was reduced in the mutant, we conducted RT-PCR analysis using primers 145S and 145AS encompassing this intron (Figure 6A). RNA preparations were obtained from wild-type and *spo14-B221* mutant, which were incubated either in growth or sporulation media. As expected, splicing of the fifth intron in *spo14-B221* cells was severely reduced either in vegetative growth or sporulation and at both permissive and restrictive temperatures (Figure 6B).

Next, the abundance of *spo14* mRNA was compared between wild-type and *spo14-B221* strains by Northern analysis. Interestingly, the *spo14* mRNA was hardly detectable in *spo14-B221* cells cultured in the medium with or without a nitrogen source (Figure 6C). Possibly, decreased efficiency of splicing may cause an instability of mRNA molecules rather than the accumulation of unspliced mRNA. In fact, Western analysis showed that Spo14 was remarkably decreased in *spo14-B221* mutant (Figure 6D). As the apparent size of Spo14 was comparable to the wild-type protein, it might be produced from the barely spliced *spo14* mRNA in the *spo14-B221* mutant. Therefore, we suspect that overexpression of *spo14-B221* should complement the sporulation defect. The transformant with a multicopy plasmid pAL(spo14-B221) sporulated well (Figure 6E) and contained approximately the wild-type level of Spo14 (Figure 6F). Because the *spo14-B221* mutant grows normally, these data also suggest that the sporulation process requires higher amounts of Spo14 protein than does vegetative growth.

The spo14-B221 Mutant Is Defective in Forespore Membrane Formation

We examined how the *spo14-B221* mutation impairs sporulation in more detail. During meiosis II, SPBs structurally change from a compact dot to a crescent (Hagan and Yanagida, 1995; Ikemoto *et al.*, 2000). However, Immunostaining of the SPB showed that modified crescent-shaped

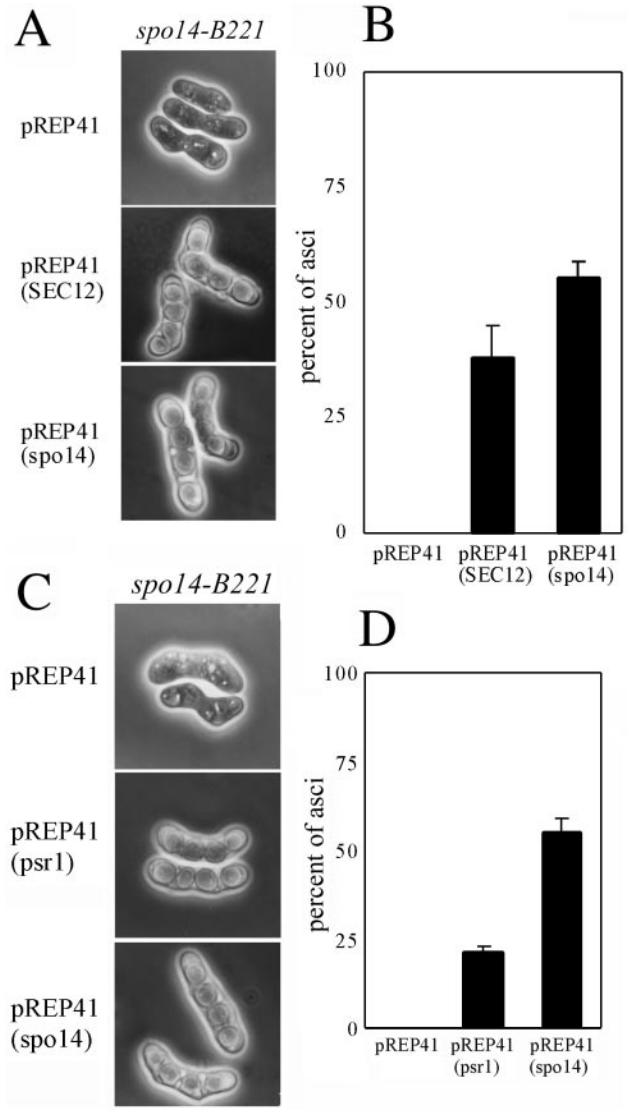

Figure 5. Spo14 is a functional homologue of Sec12. (A) Overexpression of *S. cerevisiae SEC12* suppressed sporulation deficiency of *spo14-B221*. Strain MK14L (*spo14-B221*) transformed with either pREP41, pREP41(SEC12), or pREP41(spo14) was incubated on SSA at 23°C for 5 d. (B) Sporulation frequency of the transformants. Mean of percent asci of three independent transformants is shown with SEs (vertical bars). (C) Overexpression of *psr1*$^+$ suppressed sporulation deficiency of *spo14-B221*. Strain MK14L (*spo14-B221*) transformed with either pREP41, pREP41(psr1), or pREP41(spo14) was incubated on SSA medium at 23°C for 5 d. (D) Sporulation frequency of the transformants. Mean of percent asci of three independent transformants is shown with SEs (vertical bars).

SPBs were observed in *spo14-B221* mutant cells (Figure 7B) at a frequency comparable to wild-type cells during the second meiotic division. Essentially the same results were obtained when the SPB was stained with the GFP-tagged Spo15, which is another SPB-associated protein (Ikemoto *et al.* 2000). Therefore, the sporulation defect of the *spo14-B221* mutant is not due to the failure of the SPB modification.

Next, we investigated the assembly of forespore membranes in the *spo14-B221* mutant. We have recently succeeded in tracing the assembly process of the forespore membrane using GFP-tagged Psy1, a syntaxin-like protein (Nakamura *et al.*, 2001). A *spo14-B221* strain, MK14L, transformed by pREP81(GFP-psy1) was incubated in sporulation medium. Overexpression of GFP-Psy1 did not overcome the sporulation defect of *spo14-B221*. Progression of meiosis was monitored by observing the duplication of SPBs and the elongation of spindle microtubules. At the permissive temperature (30°C), the forespore membrane normally developed to engulf haploid nuclei in *spo14-B221* cells. At a restrictive temperature (23°C), forespore membrane formation did not initiate in most of the mutant cells. At a semipermissive temperature (25°C), the membrane formation initiated near the SPBs (Figure 7, A and B), but further development of the membrane was impaired (Figures 7, A and C). In ~70% of the *spo14-B221* zygotes, abnormal forespore membranes were formed (Figures 7, C and D). In 44% of the zygotes, the forespore membrane formation was arrested (Figure 7, C, type I, and D). The rest of the zygotes contained four aggregates of GFP-Psy1 near nuclei (Figure 7C, type II, and D). Comparison of the forespore membrane assembly at semipermissive (25°C) and permissive (28°C) temperatures using another marker, Spo3-GFP, confirmed the results obtained with GFP-Psy1 (Figure 7E). These results indicate that forespore membrane formation initiates normally, but its subsequent development might be aberrant in *spo14-B221* mutants. In conclusion, Spo14 appears to be responsible for the normal construction of the forespore membrane.

spo14-B221 Cells Accumulate ER-like Membranes during Sporulation

Because *S. cerevisiae* Sec12 is involved in the vesicle transport from the ER to the Golgi apparatus, the sporulation defect might be due to the blockage of vesicle transport from the ER to the Golgi apparatus in *spo14-B221* mutant during sporulation. To confirm this possibility, we observed intracellular membranous organization by electron microscopy. The wild-type and *spo14-B221* cells were sporulated on MEA medium and then processed for freeze substitution procedures. As shown in Figure 8B, membranous structures were markedly accumulated in the peripheral region of *spo14-B221* cells, though such aberrant structures were not observed in vegetative cells. These aberrant membranes might result from a block of vesicle transport from the ER. Recently we reported that *S. pombe* Sec14 homologue (Spo20) is essential for post-Golgi protein transport (Nakase *et al.*, 2001). Aberrant membrane structures shown in *spo14* mutants have not been observed in a *spo20* single mutant (Hirata and Shimoda, 1992). As shown in Figure 8C, the *spo14-B221 spo20-KC104* double mutant also exhibited prominent peripheral membranes, indicating that the development of these membranes induced by *spo14-B221* mutation is not suppressed by the *spo20-KC104* mutation. These observations suggest that the observed peripheral membranes under sporulation conditions represent an abnormal accumulation of ER-related membranes.

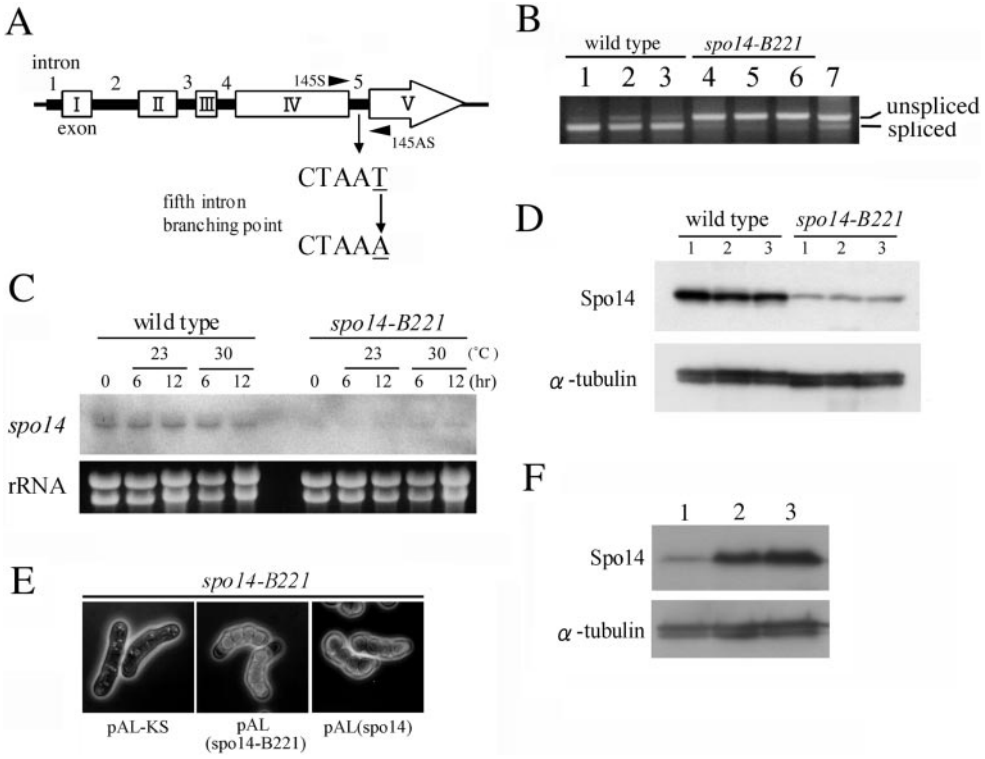

Figure 6. Analysis of the *spo14-B221* mutation. (A) Identification of the *spo14-B221* mutation point. (B) RT-PCR analysis to assess splicing of the fifth intron. The PCR primers (145S and 145AS) are shown in Figure 6A. MKW5 (wild-type) and MK14 (*spo14-B221*) cells precultured in growth medium (MM+N) at 30°C were incubated in liquid sporulation medium (MM−N) at 23 or 30°C for 10 h. Strains: lanes 1–3, MKW5; lanes 4-6, MK14. Lanes 1 and 4, 0 h at 30°C; lanes 2 and 5, 10 h at 23°C, lanes 3 and 6, 10 h at 30°C. Lane 7, same as lane 1, but without DNase treatment. (C) Northern analysis of the *spo14* mRNA in the *spo14-B221* mutant. MKW5 and MK14 cells precultured in MM+N at 30°C were incubated in MM−N at 23 or 30°C. The equality of RNA loading was confirmed by staining gels with ethidium bromide (bottom). (D) Expression of Spo14 in the *spo14-B221* mutant. MKW5 and MK14 cells precultured in MM+N at 30°C were incubated in MM−N at 23 or 30°C. Protein extracts were subjected to immunoblot analysis with the anti-Spo14 antibody, as well as anti–α-tubulin antibody as the loading control. Lane 1, 0 h at 30°C; lane 2, 10 h at 23°C; lane 3, 10h at 30°C. (E) Overexpression of *spo14-B221* complemented the sporulation defect of the *spo14-B221* mutant. Strain MK14L (h^{90} *spo14-B221*) carrying either pAL-KS, pAL(spo14-B221), or pAL(spo14) was incubated on SSA medium at 23°C for 5 d. (F) Expression of Spo14-B221 in the *spo14-B221* mutant. MK14L cells transformed with either pAL-KS (lane 1) or pAL(spo14-B221) (lane 2) and MKW5 (lane 3) were precultured in MM+N at 30°C, and then cells were incubated in MM−N for 8 h. Immunoblot analysis was carried out as described in Figure 6D.

DISCUSSION

The spo14⁺ Gene Encodes an S. cerevisiae *Sec12-like Protein and Is Required for the Protein Transport from the ER to the Golgi Apparatus*

The isolated *spo14⁺* gene was identical to *stl1⁺*, which had been identified as a gene that complements the temperature sensitivity of the *S. cerevisiae sec12* mutant (d'Enfert *et al.*, 1992). Several lines of evidence obtained in this study showed that Spo14 played a function equivalent to *S. cerevisiae* Sec12 in the protein transport pathway, as follows: 1) Spo14 localizes to the ER membrane; 2) Spo14 and *S. cerevisiae* Sec12 mutually complemented the mutational defects; 3) abnormal ER-like membrane structures were accumulated in *spo14-7* at the restrictive temperature; 4) an ER/Golgi shuttling protein Rer1 remained in the ER when conditional *spo14-7* mutants were incubated at the restrictive temperature; 5) overexpression of *psr1⁺*, an *S. pombe* homologue of *S. cerevisiae SAR1*, suppressed the cold sensitivity of *spo14-6* and *spo14-7* mutations in the same way that *SAR1* overexpression rescued *sec12* temperature-sensitive alleles in *S. cerevisiae*. Recently, Matynia *et al.* (2002) reported that *S. pombe* Psr1 is also involved in the transport from the ER to the Golgi apparatus. From these results, we conclude that *S. pombe* Spo14 plays a crucial role in membrane traffic from the ER to the Golgi apparatus, probably by regulating the Psr1 activity.

Sporulation-specific Phenotype of the spo14-B221 Mutant

As described above, sporulation deficiency of *spo14-B221* is caused by the reduction of the *spo14* mRNA level. The apparent decrease in the *spo14* mRNA could be explained by transcriptional repression of *spo14* and/or increased instability of the nascent *spo14* mRNA. Recently an idea has been proposed that transcription, splicing, capping, and addition of poly(A) tails are coupling in limited areas of the nucleus (McCracken *et al.*, 1997). According to this RNA factory model, decreased splicing efficiency results in the reduction of transcriptional activity. Alternatively, insufficient splicing of the fifth intron may cause transcript instability, because splicing intermediates are thought to be unstable. In either case, the sporulation defect of the *spo14-B221* mutant is likely to be due to the level of Spo14, implying that the sporulation process requires higher amounts of Spo14 than does the vegetative growth. This finding was further supported by the result that ER-like membrane structures are accumulated in *spo14-B221* mutant during sporulation, although the mutant can grow normally. Transcription of a number of general secretory genes of *S. cerevisiae* including

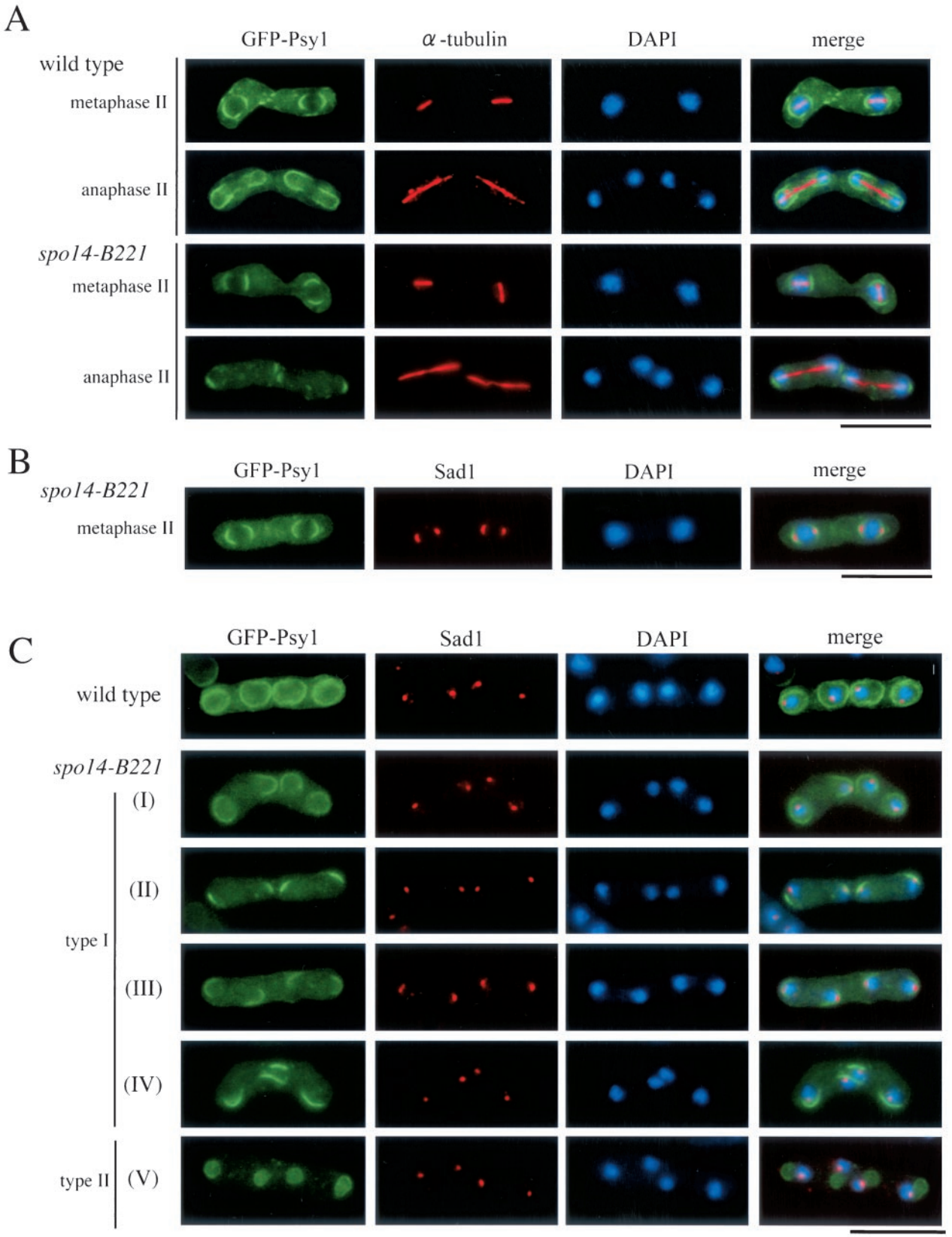

Figure 7 (facing page).

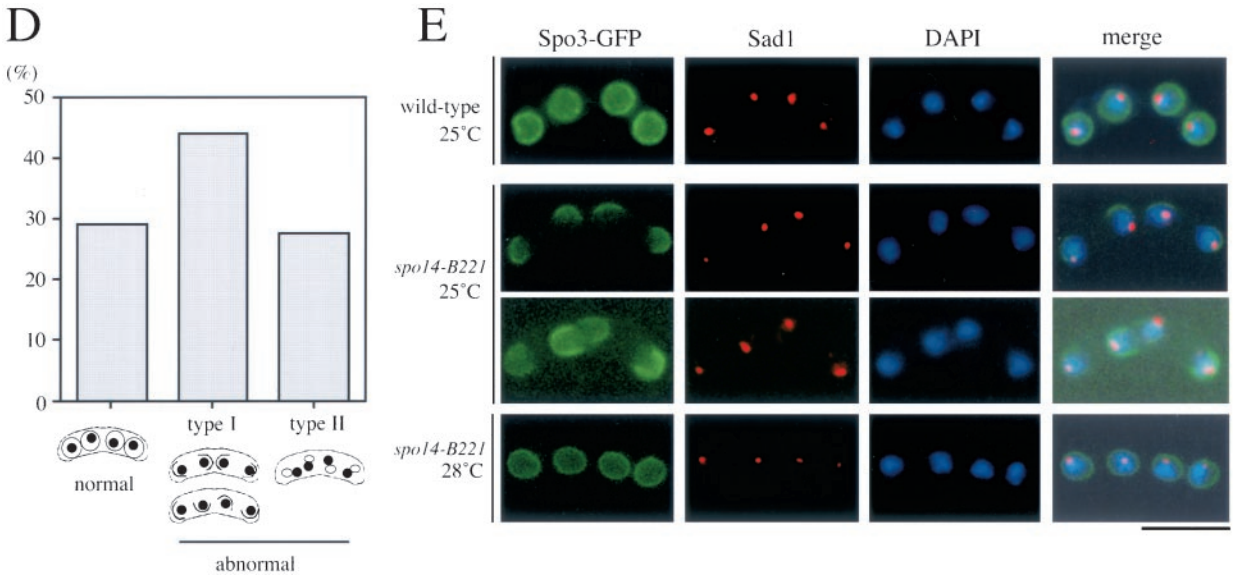

Figure 7 (cont). Aberrant assembly of forespore membranes in *spo14-B221*. (A and B) Assembly of the forespore membrane during metaphase II and anaphase II. TN8 (wild-type) and MK14L (*spo14-B221*) transformed with pREP81(GFP-psy1) were cultured in SSL−N to induce meiosis at a semipermissive temperature (25°C) for 8 h. Fixed cells were doubly stained with the anti–α-tubulin (A) or anti-Sad1 antibody (B) and DAPI. Bars, 10 μm. (C) Classification of terminal phenotypes of the forespore membrane in *spo14-B221* zygotes. Strain, culture conditions, and staining procedures are the same as described in Figure 7B. Type I, zygotes in which forespore membranes were formed at the normal site but did not extend; Type II, zygotes in which four aggregates of GFP-Psy1 were formed close to nuclei. (D) Relative frequency of the cell types of Figure 7C. Stained cells were categorized into three classes, normal, abnormal type-I and abnormal type-II. The values depicted show one representative result (N = 400) of three independent experiments. (E) Observation of forespore membrane using another forespore membrane marker, Spo3-GFP. MK14L cells were transformed with pAL(spo3-GFP) to visualize the forespore membrane by Spo3-GFP. Cells were incubated in SSL−N at either a permissive (28°C) or a semipermissive temperature (25°C). Fixed cells were doubly stained with anti-Sad1 antibody and DAPI. Bar, 10 μm.

SEC12 is further stimulated during sporulation (Chu *et al.*, 1998). We also recently demonstrated that *psy1*$^+$ encoding a syntaxin 1A-like protein is essential for vegetative growth and that its transcription is further enhanced during meiosis (Nakamura *et al.*, 2001). Requirement of sporulating cells for the general secretory machinery is explainable by the bulk de novo synthesis of the forespore membrane. The fact that reduced levels of Sec12 homologous proteins (Spo14) actually result in premature arrest of the forespore membrane supports this notion.

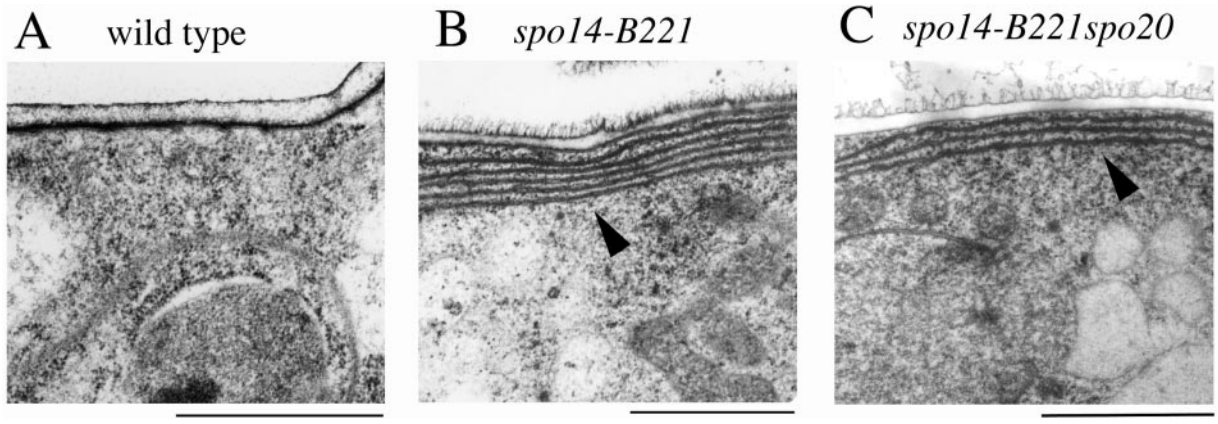

Figure 8. Accumulation of peripheral ER-like membranes during meiosis and sporulation in *spo14-B221* mutants. Cells were sporulated on SSA medium at 23°C for 24 h. Strains used are as follows: TN52 (wild-type), B221 (*spo14-B221*), and TN196 (*spo14-B221 spo20-KC104*). Arrowheads in B and C indicate ER-like membranes. Bars, 1 μm.

The Role of Spo14 in Forespore Membrane Formation

Our fluorescence microscopic observations reveal that, in both *spo14-B221* and *spo3*Δ mutants, forespore membrane formation initiates normally near the SPB during meiosis II, but subsequent development into membrane compartments containing a nucleus, called prespores, cannot be completed (this study and Nakamura *et al.*, 2001). However, the terminal phenotypes of these two mutants are different. The *spo3*Δ zygotes formed four amorphous aggregates of GFP-Psy1 near nuclei or extremely small nucleated prespores (Nakamura *et al.*, 2001). In contrast, the development of prespores in *spo14-B221* is blocked in the course of forespore membrane assembly. We speculate that Spo3 that localizes to the forespore membrane is responsible for assembly and/or integrity of the membrane, whereas Spo14 may be involved in the supply of membrane vesicles from the ER.

We reported that Spo14 is preferentially present around the nucleus and the cell periphery. Interestingly, the peripheral localization was lost during meiosis, though the localization around nuclei remained. We do not know whether this alteration is due to the Spo14-specific one or rearrangement of ER structure, because other ER markers, such as 13g6-GFP, disappeared during meiosis. An answer to this intriguing question awaits further molecular and cytological analysis.

On the basis of the present study and our previous reports (Ikemoto *et al.*, 2000; Nakase *et al.*, 2001, Nakamura *et al.*, 2001), we propose a model for construction of the forespore membrane (Figure 9). From metaphase II to anaphase II, the SPB undergoes morphological alteration to a multilayered form, depending on Spo15 (Ikemoto *et al.*, 2000). The t-SNARE protein Psy1, probably as well as a SNAP-25 homologue, is recruited to the modified SPB and is localized to nascent forespore membranes. These t-SNARE proteins on the target membrane, the precursor structure of the forespore membrane, are implicated in the fusion with small vesicles. Another forespore membrane protein Spo3 may contribute to its assembly and integrity by promoting efficient membrane fusion or stabilizing the nascent architecture. Thus, the forespore membrane extends and eventually encapsulates each of the haploid nuclei. The secretory pathway components Spo14 and Spo20 serve to supply membrane vesicles for the forespore membrane.

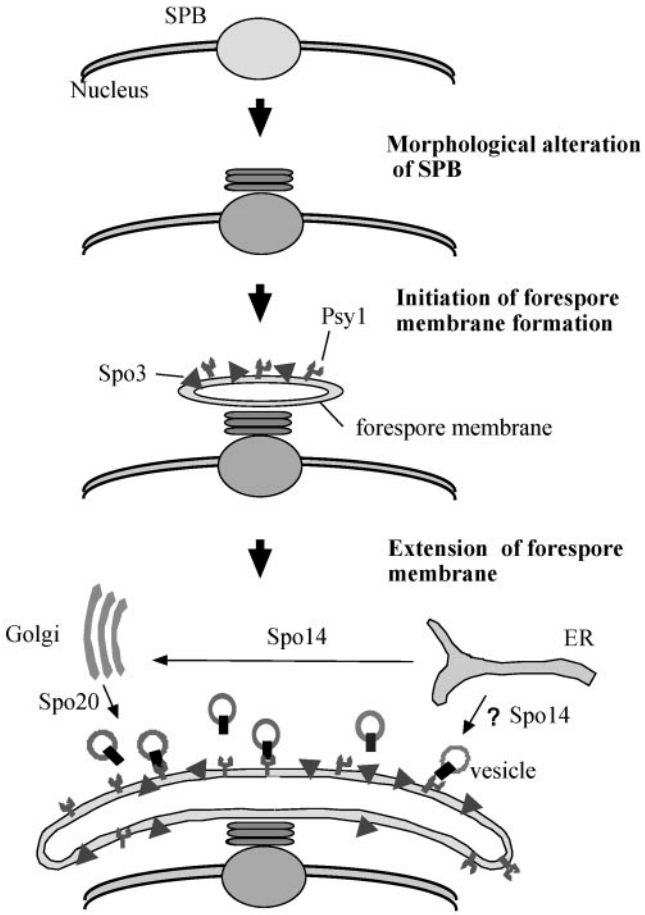

Figure 9. A model for assembly of the forespore membrane.

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