Novel Fission Yeast Cdc7-Dbf4-Like Kinase Complex Required for the Initiation and Progression of Meiotic Second Division

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Cdc7, a conserved serine/threonine protein kinase, controls initiation of DNA replication. A regulatory subunit, Dbf4, stimulates the kinase activity of Cdc7 and recruits it to the replication origins. *Schizosaccharomyces pombe* has a homologous kinase complex, composed of Hsk1 and Dfp1/Him1. Here, we report a novel protein kinase of *S. pombe*, Spo4, which shares common structural features with the Cdc7 kinases. In spite of the structural similarities, Spo4 is dispensable for mitotic growth and premeiotic DNA replication. Intriguingly, *spo4* null mutants are defective in initiation and progression of the second meiotic division. Spindles for meiosis II are often fragmented. Spo4 kinase activity is markedly enhanced when the enzyme is associated with its regulatory subunit, Spo6, a Dbf4-like protein. Expression of Spo4 is specifically induced during meiosis. Spo4 is preferentially present in nuclei, but this nuclear localization does not require Spo6. These results suggest that Spo4 is a Cdc7 kinase whose primary role is in meiosis, not in DNA replication. This is the first report of an organism which has two Cdc7-related kinase complexes with different biological functions.

The fission yeast *Schizosaccharomyces pombe* initiates a sporulation program when starved of nutrients, particularly nitrogen sources (14, 16, 71). Ascospore formation is a process of gametogenesis, because it is linked to meiotic nuclear divisions. Yeast meiosis shares a fundamental mechanism with the meiosis in higher eukaryotes. Two meiotic divisions are preceded by a round of premeiotic DNA replication. The first division is characterized by reductional chromosome segregation. Between the first and second meiotic divisions, no DNA replication occurs. The mechanism for skipping the S phase relates to the premature reactivation of maturation-promoting factors after meiosis I in *Xenopus* oocytes (20, 53), but it has not been established whether the same mechanism also exists in fission yeast. The second meiotic division is similar to mitotic division because the sister chromatids are separated, and thus it is called equational chromosome segregation (9, 15, 16, 71).

In both budding and fission yeasts, meiosis has been analyzed genetically. Many meiosis- and sporulation-deficient *S. pombe* mutants have been isolated and analyzed (5, 41). The study of genomic or cDNA clones which complement the meiosis-defective mutation has identified several meiotic genes, such as the *mei2*+ gene encoding a crucial inducer of meiosis and *mei4*+ encoding a meiosis-specific transcription factor (29, 66, 67). *S. pombe* mutants deficient in meiotic recombination have also been extensively explored. Some of the *rec*+ genes are essential for normal progression of meiosis. For example, *rec8*+ codes for the meiosis-specific cohesin and plays an essential role in sister chromatid cohesion during meiosis I (56, 68). The *spo*+ genes were originally defined as those that are not required for mitotic proliferation and meiotic division (5,

41). The traits of many *spo* genes have recently been reexamined by cloning and disruption. We have isolated *spo2*, *spo3*, *spo4*, *spo5*, *spo6*, *spo13*, *spo14*, *spo15*, *spo18*, *spo19*, and *spo20* (31, 51; M. Nakamura-Kubo, T. Nakamura, and C. Shimoda, unpublished data). At least two *spo* genes (*spo14*+ and *spo20*+) were shown to be necessary not only for sporulation but also for vegetative growth (52; Nakamura-Kubo et al., unpublished). *spo20*+ encodes a structural and functional homologue of budding yeast Sec14 (52). The *Saccharomyces cerevisiae* Sec14 is a phosphatidylinositol transfer protein and is crucial to post-Golgi vesicle traffic (39). Furthermore, some *spo* mutants fail to undergo sporulation as a consequence of a defect in meiosis. The *spo6*+ gene regulates the progression of meiosis II, because most *spo6* mutant cells fail to complete meiosis II (50). Interestingly, the predicted Spo6 protein has high similarity to budding yeast Dbf4, which is a regulatory subunit of Cdc7 serine/threonine kinase (32, 36, 42). Thus, it is likely that an *S. pombe* Cdc7 homologue is involved in meiosis and sporulation.

The *S. cerevisiae* Cdc7 kinase is composed of Cdc7 (a catalytic subunit) and Dbf4 (a regulatory subunit). A Cdc7-Dbf4 kinase complex controls initiation of DNA replication (4, 12, 13, 27, 28, 35, 43, 57, 62, 63). Like Cdks, Cdc7 kinase activity is regulated by association with Dbf4 (32, 42). The abundance of Dbf4 periodically fluctuates, peaking at S phase (10, 11, 17, 55), while Cdc7 levels remain constant during the cell cycle (61). The Cdc7-Dbf4 kinase complex is evolutionarily conserved among eukaryotic organisms (34, 35, 37, 40, 43, 46, 59). The fission yeast *S. pombe* has a homologous kinase complex, composed of Hsk1 and Dfp1 (also known as Him1), which is also essential for the onset of DNA replication (6, 7, 45, 63).

Spo6 and Dfp1 are encoded by different genes, indicating that *S. pombe* has two Dbf4-like proteins. *spo6*+ is dispensable for both mitotic and premeiotic DNA synthesis. Spo6 and Hsk1 show no physical interaction. These findings suggest that

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TABLE 1. Strains used in this study

Strain	Genotype	Source
S. pombe		
L968	h[90]	U. Leupold
B4	h[90] *spo4-B4 ade6-M210*	5
TN29	h[90] *leu1-32 ura4-D18*	31
TN47	h[+s] *ade6-M216*	51
TN53	h[−] *ade6-M210*	51
MK4L	h[90] *spo4-B4 leu1-32*	This study
TN33	h[90] *spo4Δ::ura4+ leu1-32 ura4-D18*	This study
HA46-11B	h[90] *spo6Δ::ura4+ leu1-32 ura4-D18*	H. Asakawa
TN68	h[+s] *spo4Δ::ura4+ ade6-M216 ura4-D18*	This study
TN69	h[−] *spo4Δ::ura4+ ade6-M210 ura4-D18*	This study
TN72	h[+s] *spo6Δ::ura4+ ade6-M210 ura4-D18*	This study
TN73	h[−] *spo6Δ::ura4+ ade6-M216 ura4-D18*	This study
TN92	h[+s] *spo4Δ::ura4+ spo6Δ::ura4+ ade6-M210 ura4-D18*	This study
TN93	h[−] *spo4Δ::ura4+ spo6Δ::ura4+ ade6-M216 ura4-D18+*	This study
JZ670	h[−]/h[−] *pat1-114/pat1-114 ade6-M210/ade6-M216 leu1-32/leu1-32*	M. Yamamoto
AB4	h[−]/h[−] *mei4Δ::ura4+/mei4Δ::ura4+ pat1-114/pat1-114 ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*	1
TN74	h[+s]/h[−] *spo4Δ::ura4+/spo4Δ::ura4+ ade6-M210/ade6-M216 ura4-D18/ura4-D18*	This study
TN75	h[+s]/h[−] *ade6-M210/ade6-M216*	This study
TN76	h[+s]/h[−] *spo6Δ::ura4+/spo6Δ::ura4+ ade6-M210/ade6-M216 ura4-D18/ura4-D18*	This study
TN77	h[+s]/h[−] *spo4Δ::ura4+/spo4Δ::ura4+ spo6Δ::ura4+/spo6Δ::ura4+ ade6-M210/ade6-M216 ura4-D18/ura4-D18*	This study
TN151	h[−]/h[−] *spo4Δ::ura4+/spo4Δ::ura4+ pat1-114/pat1-114 ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*	This study
TN188	h[−]/h[−] *pat1-114/pat1-114 ade6-M210/ade6-M216 leu1-32/leu1-32 spo4-HA≪LEU2/spo4+*	This study
TN194	h[90] *spo4-HA≪LEU2 leu1-32*	This study
TN195	h[90] *spo4-HA≪LEU2 spo6Δ::ura4+ leu1-32 ura4-D18*	This study
TN229	h[90] *spo4-B4 leu1-32 ura4-D18*	This study
S. cerevisiae		
Y187	*matα ura3-52 his3-200 ade2-101 trp1-901 leu2-3, 112 gal4Δ mef gal80Δ URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ*	26

the partner of Spo6 is not Hsk1 but a novel Cdc7-like kinase (50). In this article, we report a gene termed *spo4+*, which encodes a second Cdc7-like protein. Spo4 forms an active kinase complex with Spo6. Unlike known Cdc7 kinases of budding yeast and other eukaryotes, *spo4+* is essential for neither mitotic nor premeiotic DNA replication. However, *spo4+* is required for the initiation and progression of a second meiotic division. In addition, *spo4+* activity is regulated at the transcriptional level. We discuss a model in which Spo4 regulates the initiation and progression of meiosis II.

MATERIALS AND METHODS

Yeast strains, media, and culture conditions. The *S. pombe* strains used in this study are listed in Table 1. A sporulation-deficient *spo4-B4* mutant of *S. pombe* was isolated by Bresch et al. (5) and genetically analyzed by Kishida and Shimoda (41). The complete medium YEA supplemented with 75 μg of adenine sulfate/ml and 50 μg of uracil/ml was used for growth. The synthetic medium MM was used. The malt extract medium MEA and the synthetic sporulation media SSA, SSL−N, and MM−N were used for mating and sporulation. These media were

described by Egel and Egel-Mitani (15), Gutz et al. (22), and Moreno et al. (49). *S. pombe* cells were grown and sporulated at 28°C.

Cloning of *spo4+*. A homothallic *spo4-B4* mutant (MK4L) was transformed with an *S. pombe* genomic library containing partial *Sau*3AI fragments (a gift from Y. Watanabe) constructed in a multicopy plasmid, pDB248′ (3), and with a cDNA library, pTN-RC5 (this study), containing meiotic cDNA fragments constructed in the expression vector pREP42 (47). One of 15,000 transformants from the genomic library and four of 100,000 transformants from the cDNA library were able to sporulate. Plasmid DNA molecules were recovered from these transformants, and the plasmids obtained from the genomic library were further analyzed.

Plasmid construction. Plasmid pIL2 was constructed as follows. A 2.2-kb fragment containing *LEU2* was ligated into the *Ssp*I site of pBluescript KS(−) to create pIL2. A 2-kb *Not*I-*Sac*I fragment, which contains three tandem repeats of the hemagglutinin (HA) epitope and the *nmt1* terminator of pSLF272 (19), was ligated into the same sites of pIL2 to create pTN218. pREP41(*Not*I) was constructed by inserting a *Not*I linker into the *Sma*I site of pREP41. Two oligonucleotides, spo4N and pREP-AS (see below), were used to amplify *spo4+* cDNA. As a template, pREP42(spo4), containing *spo4+* cDNA, was used. The 1.4-kb fragment was digested with *Sal*I and *Not*I and inserted into the corresponding site of pREP41(*Not*I) and pTN218, yielding pREP41(spo4) and pIL2(spo4-HA), respectively. pREP41(GST) was constructed by inserting the *Pst*I-*Sac*I fragment of glutathione *S*-transferase (GST) from pDS473 (19) into the same site of pREP41. For expression of GST-Spo4, pREP41(GST-Spo4) was used. This plasmid was constructed by inserting the full length of *spo4+* cDNA into plasmid pREP41(GST). pTN63 was constructed using two oligonucleotides, 5′-GGCCG CGACTATAAGGACGACGATGACAAGTGACCGCGG-3′ and 5′-TCGACC GCGGTCACTTGTCATCGTCGTCCTTATAGTCGC-3′. pREP42(spo4) was used as a template. These DNAs were annealed and inserted into the *Not*I-*Sal*I site of pSLF272 (19), yielding pTN63. For expression of Spo6-FLAG, pTN63(spo6) was used. This plasmid was constructed as follows. Two oligonucleotides, 5′-CCCCTCGAGTATGGACTTCTATTCAGTGAAG-3′ (the *Xho*I site is underlined) and 5′-GATGCGGCCGCCATTTGTCCGAATTGGGCG-3′ (the *Not*I site is underlined), were used to amplify the *spo6+* cDNA by PCR. pREP42(spo6), containing *spo6+* cDNA, was used as a template. The PCR product was digested with *Xho*I and *Not*I and then inserted into the same site of pTN63, yielding pTN63(spo6). For two-hybrid analysis, a DNA fragment encoding full-length *spo4+* cDNA was cloned into pGAD424 to express Spo4 fused with the Gal4 transcriptional activator domain pGAD(spo4). A DNA fragment encoding full-length *spo6+* cDNA was cloned into pGBT9 to express Spo6 fused with the Gal4 DNA binding domain (50).

Disruption of spo4. *spo4+* was disrupted by inserting *ura4+* into its coding region. A 4.9-kb *Sph*I-*Sal*I fragment was subcloned into pHSG396 (TaKaRa). A 0.9-kb *Hind*III-*Stu*I fragment was replaced with the 1.8-kb *ura4+* cassette (21) (Fig. 1B). The 5.8-kb *Hind*III-*Sal*I fragment containing the interrupted *spo4* allele (*spo4::ura4+*) was used to transform the strain, TN29. Disruption was confirmed by Southern hybridization of genomic DNA (data not shown).

Site-directed mutagenesis. The four kinds of mutation (K95A, D182N, T264A, and T264E) in the *spo4* gene were created by PCR with the following primers: for the N-terminal fragment, 5′-CCCGTCGACAATGCTTTCCATGCTATTAC C-3′ (spo4N) (the *Sal*I site is underlined), 5′-CGGTGGCATAAATTCTGGCC AATGCAACAAAATGGTTTTTTG-3′ (K95A-as), 5′-GTTACCTGGTTTGA TATTTCGATGAATTATGCCCTTTGCATC-3′ (D182N-as), 5′-CGAAATCC TCTTGCTCCGGCTCGGTCAGCA-3′ (T264A-as), and 5′-CGAAATCCTCT TTCTCCGGCTCGGTCAGCA-3′ (T264E-as); for the C-terminal fragment, 5′-GTTGCATTGGCCAGAATTTATGCCACCGTTCTTCCC-3′ (K95A-s), 5′-G GGCATAATTCATCGAAATATCAAACCAGGTAACTTTGC-3′ (D182N-s), 5′-GACCGAGCCGGAGCAAGAGGATTTCGAGCG-3′ (T264A-s), 5′-GAC CGAGCCGGAGAAAGAGGATTTCGAGCG-3′ (T264E-s), and 5′-CAAAAT CGTAATATGCAGCTTGAATGGGC-3′ (pREP-AS). The mutated alleles were obtained by PCR with spo4N–K95A-as and K95A-s–pREP-AS for K95A, spo4N–D182N-as and D182N-s–pREP-AS for D182N, spo4N–T264A-as and T264A-s–pREP-as for T264A, and spo4N–T264E-as and T264E-s–pREP-as for T264E. In the PCRs, pREP42(spo4) was used as a template. In every case, the fragments produced were subjected to a second PCR with a pair of primers, spo4N and pREP-AS. The products were restricted by *Sal*I and *Not*I, and the resulting 1.4-kb fragment was inserted into the same site of pREP41(*Not*I) and pREP41(N-HA), yielding pREP41(spo4[K95A]) and pREP41(HA-spo4[K95A]), pREP41(spo4[D182N]) and pREP41(HA-spo4[D182N]), pREP41(spo4[T264A]) and pREP41(HA-spo4[T264A]), or pREP41(spo4[T264E]) and pREP41(HA-spo4[T264E]), respectively. Plasmid pREP41(GST-spo4[K95A]) was constructed by inserting the *Sal*I-*Not*I fragment of pREP41(spo4[K95A]) into the corresponding site of pREP41(GST).
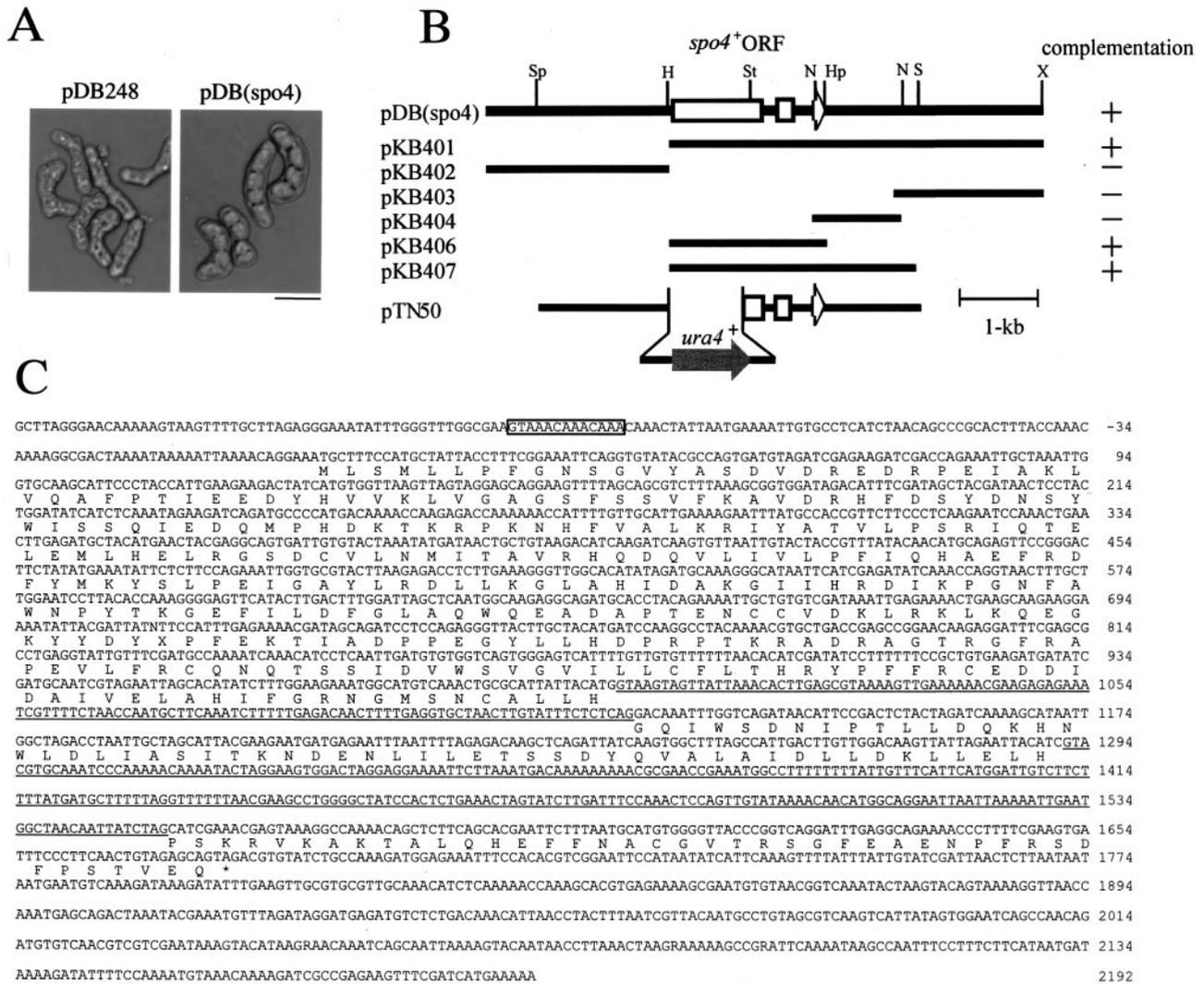
FIG. 1. Isolation, subcloning, and sequencing of the *spo4+* gene. (A) Isolation of the *spo4+* gene. Strain MK4L (*spo4-B4*) was transformed with either pDB248′ or pDB(spo4). The Leu+ transformants were incubated on synthetic sporulation medium (SSA) at 28°C for 2 days. (B) Restriction map and construction of null mutants. The open arrows indicate the region and direction of the *spo4+* open reading frame, which encodes a protein composed of 429 amino acid residues. All subclones were derived from pDB(spo4). Complementation by each subclone is indicated as follows: +, complements; −, does not complement. Restriction enzyme sites: H, *Hin*dIII; Hp, *Hpa*I; N, *Nsi*I; S, *Sal*I; Sp, *Sph*I; St, *Stu*I; X, *Xho*I. (C) Nucleotide and predicted amino acid sequences of *spo4+*. Two introns are marked by underlining. A putative FLEX sequence in the promoter region is boxed.

Southern and Northern analyses. Genomic DNA was restricted, fractionated in a 1.0% agarose gel, and then transferred to nylon membranes (Biodyne A; Nihon Pall Co). Total RNA was prepared from *S. pombe* cultures (33) and fractionated on a 1.0% gel containing 3.7% formaldehyde as previously reported (65).

Western blotting. The pIL2(spo4)-HA plasmid was linearized by restricting it with *Stu*I near the center of the *spo4* sequences and was introduced into TN8. Since Leu+ transformants which were competent for sporulation were obtained, we concluded that Spo4-HA is a functional protein. Likewise, this plasmid was integrated into HA46-11B and JZ670. A wild-type strain (TN194) and a *spo6* mutant strain (TN195) were cultured in liquid sporulation medium (SSL−N). At intervals, culture aliquots were collected and crude cell extracts were prepared as described by Masai et al. (45). Polypeptides were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and then transferred to polyvinylidene difluoride membranes (Millipore). The filters were probed with either mouse anti-HA antibody 12CA5 (Boehringer Mannheim), mouse anti-FLAG M2 antibody (Sigma), rat anti-HA antibody 3F10 (Boehringer Mannheim), or goat anti-GST antibody (Pharmacia) at a 1:1,000, 1:1,000, 1:1,000, or 1:200 dilution, respectively. Blots were also probed with anti-α-tubulin

antibody, TAT-1 (70), to normalize the protein load. Immunoreactive bands were visualized by staining them with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; Promega), goat anti-rat IgG (BioSource), or rabbit anti-goat antibody (ICN/CAPPEL) and by chemiluminescence (NEN Life Sciences).
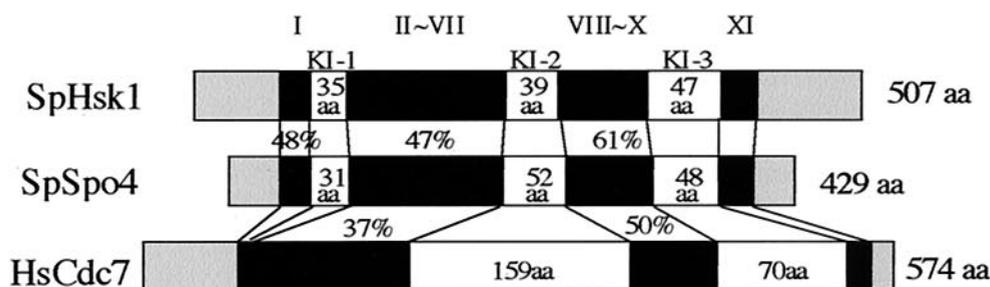
Immunofluorescence microscopy. For cell fixation, we followed the procedure of Hagan and Hyams (24), using glutaraldehyde and paraformaldehyde. The spindle pole body (SPB) was visualized by indirect immunofluorescence microscopy using rabbit anti-Sad1 antibody (a gift from O. Niwa) and Alexa 546-conjugated goat anti-rabbit IgG (Molecular Probes) (23). For microtubule staining, TAT-1 anti-α-tubulin antibody (70) and Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes) were used. Spo4-HA was visualized using rat anti-HA antibody 3F10 and Alexa 488-conjugated goat anti-rat IgG (Molecular Probes). The nuclear chromatin region was stained with 4′,6-diamidino-2-phenylindole (DAPI) at 1 μg/ml. Stained cells were observed under a fluorescence microscope (model BX50; Olympus) and a Cool SNAP charge-coupled device camera (Roper Scientific).

Interaction of GST-Spo4 with Spo6-FLAG in *S. pombe* cells. The strain TN29 was cotransformed with either pREP41(GST), pREP41(GST-spo4), or pREP

A

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                    I                      Kinase Insert I                   II          III
SpSpo4  ------------------------------------MLSMLLPFGNSGVYASDVDREDRPEIAKEVQKFETIEEDMHVVKLV   46
SpHsk1  -MAEAHITLSPKVTHEQQTDIDSECEITEVDDENVNENKSQEMIQDIPARDREEIENITRTFVELQENYRLIEKI            74
ScCdc7  -----------------------------MTSKTKNIDDIPPEIREEMIQLYHDLEGIENEEKLIDKI                   39
MuCdc7  -----------------MEEPMAFSSLRG-SDRCPADDSLKKYESVKLSGIKRDFEELGEAVEQLVNVSKIKDKI            58
HsCdc7  --------------MEASLGIQMDEPMAFSPQR---DRFQAEGSLKKNEQNFKLAGVKDKLYEAVEQLSNVFKIEDKI         64

SpSpo4  GAGSFSSVFKAVDRHFDSYDNSYWISSQIEDQMPHDKTKR------PKNHFVALKRIYATVLFSRIQTEIEMEHE           115
SpHsk1  GEGTFSSVYKAELHYGRYINDWDIQSEVLKESSFGKEKIPVNEDSRKPKYVAIKKIYATSSEARIYNELEIIYL           149
ScCdc7  GEGTFSSVYKAKDITGKITKKFASHFWN-------------------YGSNYVALKKIYATSSPQRIYNELNLYI            96
MuCdc7  GEGTFSSVYLATAQLQE-----------------------------GHEEKIALKHLIPTSHEMRIAAELQCLTV          104
HsCdc7  GEGTFSSVYLATAQLQV-----------------------------GPEEKIALKHLIPTSHEIRIAAELQCLTV          110

                 IV                    V                               VI
SpSpo4  LRGSDCVLNMITTVRHQDQVLIVLEFIQHAEFRDFVMKYSLPEIGAHLRDELKGIAHIDAKGIIHRDIKPGNFAW          190
SpHsk1  LRGSSVIAPLIITALRNEDQVLVVLPYYEHTDFRQYVSTFSYRDMSIHFRCELEQAMQQTQTLGIIHRDIKPSNFLF        224
ScCdc7  MTGSSRVAPLCDDKEVRDNDVVLAMPYYPHEDFRTFYRDLPIKGIKKHIWELLRALKFVHSKGIIHRDIKPTNFLF        171
MuCdc7  AGGQDNVMGLKYCFVRKNDHVVLAMPYLEHESFLDILNSFSFQEVREMYNLFVALKRIHQFGIVHRDVKPSNFLY         179
HsCdc7  AGGQDNVMGVKYCFRKNDHVVLAMPYLEHESFLDILNSLSFQEVREMMLNLFVALKRIHQFGIVHRDVKPSNFLY         185

             VII                                      Kinase Insert II
SpSpo4  NPYTQRGVILDFGLAQ-------------WQEADAPTENCCVDK--------------------LR-------------      223
SpHsk1  DVRLKHGVLVDFGLAER-------------YDGRQQSHSGRCTN-----                                    255
ScCdc7  NLELGRGVLVDFGLAEAQMDYKSMISSQNDYDNYANTNHDGG-----                                     213
MuCdc7  NRRLKKYAVLDFGLAQGTRDTKIELLKFVQSEAQQEKSRNKYHGVVGHKGLLSRPAPKTVDQQCTPKTSVKRSY         254
HsCdc7  NRRLKKYALVDFGLAQGTHDTKIELLKFVQSEAQQEKSRCSQNKSHIITGNKIPLSGPVPKELDQQSTTKASVKRPY      260

SpSpo4  -------------------------------------------KLKQEGKYYDYFPFEKTIADPPEGYLLHD-           252
SpHsk1  ---------------------------------------SNAAELAHDFSIAQETSLGYIKND-                    279
ScCdc7  -------------------------------YSMRNHEQFCPCIMRNQYSPNSHNQTPPMVTIQNGKVVHLNNVN         257
MuCdc7  T--QVHIKQGKDGKERSVGLSVQRSVFGERNFNIHSSISHESPAEKDIKQSRTVDIISRKLATKKTAISTKAMN          326
HsCdc7  TNAQIQIKQGKDGKEGSVGLSVQRSVFGERNFNIHSSISHESPAVKDMKQSRTVDVLSRKLATKKKAISTKVMNS        335

                                              VIII                     IX
SpSpo4  -------------------------P-----RPTKRADRAGTRGFRAPEVLFRCQNQTSSLDVKSVGVILLCFLTH        297
SpHsk1  --------------------T-----P-----RPSKRANRAGTRGFRAPEVLFKCSSQSPKVDIWSAGVILLSFLTK       325
ScCdc7  G------------VDLTKGYPKNETRRIKRANRAGTRGFRAPEVLMKCGAQSTKIDIWSVGVILLSLLIGG             315
MuCdc7  SVMRETARSCPAVLTCDCYGSNRVCSVCLSRRQQVLPRAGTPGFRAPEVLTKCPDQTTAIDMWSAGVIFLSLLSG        401
HsCdc7  AVMRKTASSCPASLTCDCYATDKVCSICLSRRQQVLPRAGTPGFRAPEVLTKCPNQTTAIDMWSAGVIFLSLLSG        410

             X                                    Kinase Insert III
SpSpo4  RYPFFRCEDDIDAIVELAHIFGRN------GMS-NCAELHGQIWSDNIPTLLDQKHNWLDEIASITKN-------         359
SpHsk1  RFPFENSKDDVDALMEIACIFGKSE-------MRQCAALHGCTFETNVSTLTEKRVNFRKEILWASCG-----SA        387
ScCdc7  RFEMEQSLDDASELELCTIFGWKELRKCHALHGLGFEASGLIWDKPNGYSNGLKEFVYDELNKECTIG----T         385
MuCdc7  RYPFYKASDDLTALAQIMTIRGSRETIQAAKAFGKSVICSKEVPAQDLRAICERLRGLDSTTPRSASGPPGNASY       476
HsCdc7  RYPFYKASDDLTALAQIMTIRGSRETIQAAKTFGKSILCSKEVPAQDLRKLCERLRGMDSSTPKLTSDIQGHASH       485

                                                                               XI
SpSpo4  D----ENLILETSSDYQVALAIDLLDKELELHPSKRVKAKTALQHEFFNACGVTRSGFEAENPFRSDFESTVEQ-        429
SpHsk1  S-IYKEKLRHKPS--QEERLCLDFLEKCLELDCNKRISAEEALDHDFLYLDNLAYEKKDDDTAFDNSFGETSFEK        460
ScCdc7  FPEYSVAFETFGFLQQELHDRMSIEPQLPDPKTNMDAVDAYELKKYQEEIWSDHYWCFQVLEQCFEMDEQKRSSF       460
MuCdc7  DEAASKNTDHKAS-RVQAAQAQHSEDSLYKRDNDGYWSHPRDCTSNSEGWDSVPDEAYDLLDKLLDLNPASRITA       550
HsCdc7  QEAISEKTDHKASCLVQTPPGQYSGNSFKKGDSNSCEHCFDEYNTNLEGWNEVPDEAYDLLDKLLDLNPASRITA       560

SpSpo4  ----------------------------------------------                                     429
SpHsk1  DEDLITAKHLSHILDFKEQEETDEPTSLSKRKRSIDEILPNDALQDGA                                   507
ScCdc7  EDLLKTPFFNELNENTYLLDGESTDEDDVVSSSEADLLDKDVLLISE                                    507
MuCdc7  EAALLHVFFKDMCS-----                                                                564
HsCdc7  EEALLHPFFKDMS-----                                                                 573
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B

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                    I        II~VII          VIII~X           XI
                    KI-1       KI-2            KI-3
SpHsk1     [====][■|35 aa|■■■■■|39 aa|■■■|47 aa|■■====]              507 aa
                48%      47%              61%
SpSpo4     [===][■|31 aa|■■■■|52 aa|■■|48 aa|■■===]                  429 aa
                   37%                50%
HsCdc7  [====][■■■■■|  159aa  |■■■|  70aa  |■===]                    574 aa
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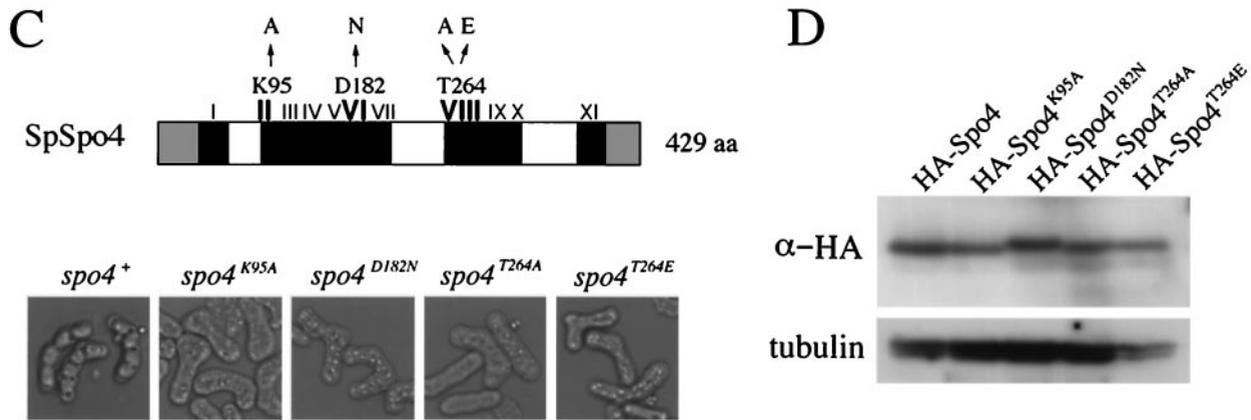
FIG. 2. *spo4*[+] encodes a novel protein which has a high degree of similarity to Cdc7-related kinase. (A) Comparison of the amino acid sequences of Spo4 and fission yeast Hsk1 (SpHsk1; GenBank accession number D50493), budding yeast Cdc7 (ScCdc7; GenBank accession number M12624), mouse Cdc7 (MuCdc7; GenBank accession number AB018574), and human Cdc7 (HsCdc7; GenBank accession number NM003503). Dark and light shaded boxes indicate identical and similar residues in three of five proteins, respectively. Dashes represent gaps in the sequences. (B) Structural similarity of Spo4 with two other Cdc7-related kinases. I to XI represent kinase subdomains. KI-1 to KI-3 represent kinase inserts. The percentages of identical amino acids (aa) in the respective regions are also shown. (C) Four *spo4* mutations and their function effects on sporulation. (Top) Positions of point mutations. All the point mutants (K95A, D182N, T264A, and T264E) contain amino acid substitutions in the kinase domains. (Bottom) Strain TN33 bearing either pREP41(spo4), pREP41(spo4^{K95A}), pREP41(spo4^{D182N}), pREP41(spo4^{T264A}), or pREP41(spo4^{T264E}) was sporulated on SSA medium for 2 days at 28°C. Bar, 10 μm. (D) Expression of *spo4* mutant proteins. Strain TN33 bearing either pREP41(HA-spo4$^+$), pREP41(HA-spo4^{K95A}), pREP41(HA-spo4^{D182N}), pREP41(HA-spo4^{T264A}), or pREP41(HA-spo4^{T264E}) was sporulated in SSL-N medium for 8 h at 28°C. Protein extracts were subjected to Western blot analysis with the rat anti-HA (α-HA), as well as with anti-α-tubulin (tubulin) antibody, as the loading control.

(GST-spo4K95A) and pREP42(spo6-FLAG). Transformants were grown in liquid MM to mid-log phase. Isolations of GST-Spo4 and Spo6-FLAG were performed according to the method described by Brown and Kelly (6).

Kinase assays using purified GST-Spo4. Either GST-Spo4 or GST-Spo4^{K95A} was expressed in wild-type cells with or without Spo6-FLAG and purified using glutathione beads from wild-type cells carrying the appropriate plasmid according to the method described by Brown and Kelly (6). Kinase reactions were performed as described previously (6) using a recombinant *S. pombe* Mcm2 protein (18, 48). To express His$_6$-Mcm2 fusion protein in *Escherichia coli*, a DNA fragment encoding amino acids 1 to 220 of Mcm2 (63) was amplified by PCR and cloned into pQE30 (Qiagen). The fusion protein was purified using Ni-nitrilotriacetic acid resin as directed by the manufacturer (Qiagen).

Nucleotide sequence accession number. The sequence data for *spo4*$^+$ are available from EMBL-GenBank-DDBJ under accession no. AB036342 (for the genome) and AB036343 (for the cDNA).

RESULTS

spo4$^+$ encodes a novel Cdc7-like protein. To identify the *spo4*$^+$ gene product and its biological function, we isolated *spo4*$^+$ by functional complementation. From a screen of about 15,000 colonies transformed with an *S. pombe* genomic library constructed in pDB248′ (3), we recovered one plasmid which rescued the sporulation deficiency of the *spo4-B4* mutant (Fig. 1A). Subcloning indicated that the complementing activity of the plasmid was associated with the 2.0-kb *Hind*III-*Hpa*I fragment (Fig. 1B). Sequencing of this fragment of pKB406 revealed one open reading frame of 1,287 nucleotides split by two potential introns (Fig. 1C). The same gene was isolated in independent complementation screens using a cDNA library (see Materials and Methods). A comparison of both genomic and cDNA sequences confirmed two introns of 123 and 261 bp (Fig. 1C). The cloned DNA fragment complementing *spo4* mutation carries the genetically defined *spo4*$^+$ itself as described below. BLAST searches showed that the predicted

Spo4 protein had significant sequence similarity to budding yeast Cdc7 kinase, fission yeast Hsk1 kinase, and mammalian Cdc7-related kinases (34, 40, 45, 58, 59) (Fig. 2A). The sequence similarity is striking in most of the kinase subdomains. Moreover, Spo4 contains three "kinase insert" stretches, which is characteristic of Cdc7-related kinases (Fig. 2B). The positions of insertion are identical to those of other Cdc7-related kinases, although mammalian Cdc7 does not have kinase insert 1 (34, 40, 59) (Fig. 2C). Budding yeast Cdc7 and fission yeast Hsk1 contain a C-terminal region rich in acidic amino acid residues, which seems essential for interaction with their regulatory subunit, Dbf4 (45, 54, 58). We note that Spo4 lacks this "acidic tail," as do the human and *Xenopus* Cdc7 homologues (34, 40, 59) (Fig. 2B). These structural features imply that Spo4 is a novel Cdc7 family kinase.

Spo4 is essential for initiation and progression of meiosis II. To investigate the biological role of *spo4*$^+$, a null mutant was created by conventional gene disruption using *ura4*$^+$ as a marker (Fig. 1B). The *spo4* deletion mutant (*spo4*Δ) was viable but displayed sporulation defects like the original *spo4-B4* mutant (data not shown). In various organisms, the Cdc7-related kinases are required for the initiation of DNA replication, suggesting that Spo4 is necessary for the initiation of premeiotic S phase. To test this possibility, we conducted flow cytometric analyses of propidium iodide-stained *pat1-114* cells that synchronously underwent meiosis at a restrictive temperature (30). The DNA content of *spo4*Δ cells roughly doubled prior to meiotic nuclear division (Fig. 3A). We observed a similar result with *spo4*Δ homozygous diploid cells cultured in nitrogen-free medium for the induction of meiosis (data not shown). We thus conclude that *spo4*Δ cells complete premeiotic DNA replication normally.
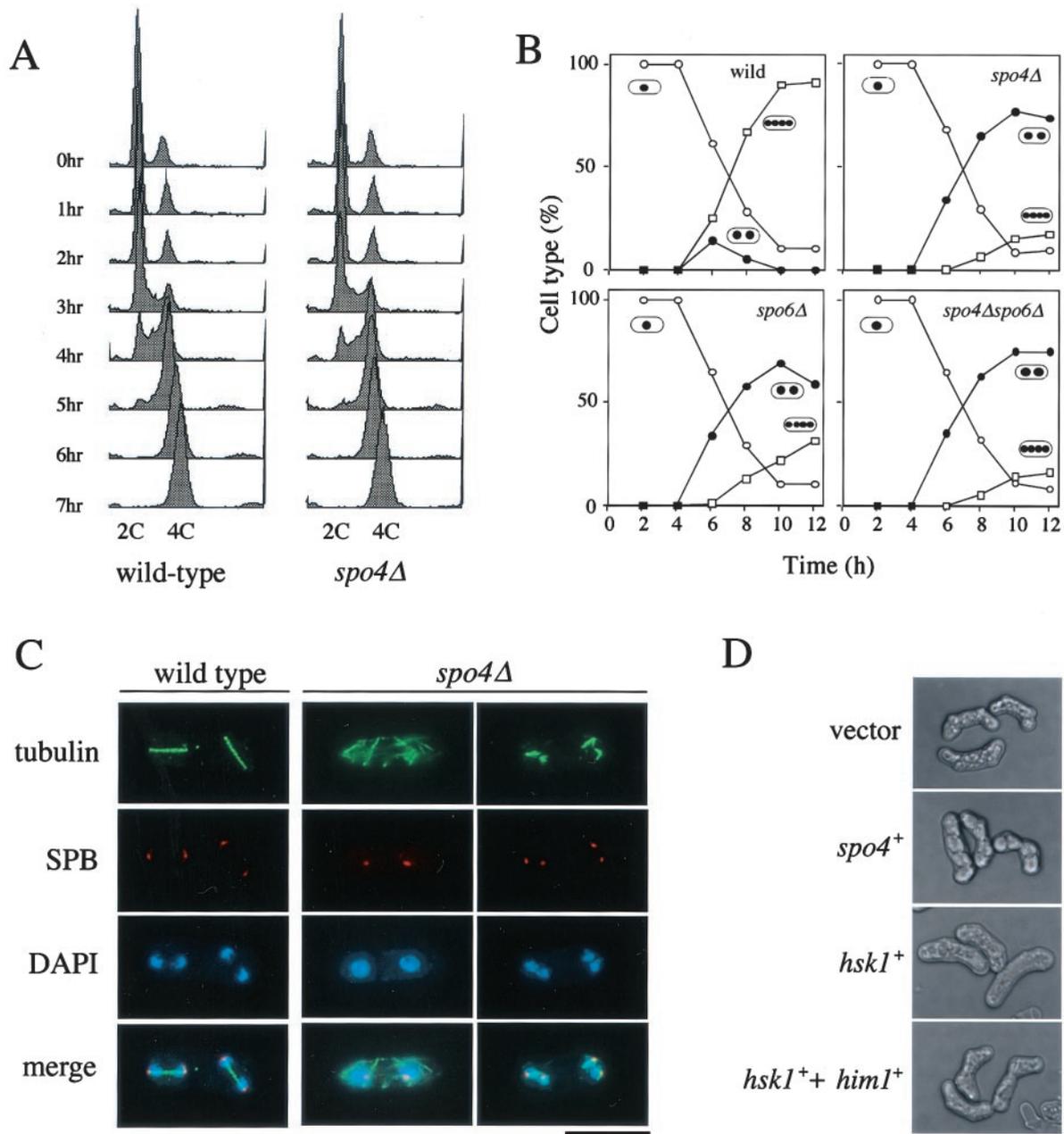
FIG. 3. $spo4^+$ is required for progression of meiosis II but not for the onset of premeiotic DNA synthesis. (A) Fluorescence-activated cell sorter analysis of premeiotic DNA replication in $spo4\Delta$ cells. Synchronous meiosis is induced in the diploid strains JZ670 ($pat1$-114/$pat1$-114) (wild type) and TN151 ($spo4\Delta$ $pat1$-114/$spo4\Delta$ $pat1$-114) ($spo4\Delta$). Samples were taken every hour, and the DNA content was measured by FACScan. (B) Kinetics of meiosis in various strains. Mid-log-phase cells of wild-type (TN75), $spo4\Delta$ (TN74), $spo6\Delta$ (TN76), and $spo4\Delta$ $spo6\Delta$ (TN77) homozygous diploid strains were cultured in MM-N medium at 28°C. The progression of meiosis was monitored by DAPI staining. One, two, and four dots in ovals represent mononucleate, binucleate, and tetranucleate cells, respectively. (C) Microtubules during meiosis visualized by immunofluorescence microscopy. Diploid cells of the wild-type (TN75) and $spo4\Delta$ (TN74) strains were incubated in SSL-N sporulation medium at 28°C, sampled, and fixed. Indirect immunofluorescence microscopy was conducted using an anti-α-tubulin and an anti-Sad1 antibody to visualize microtubules and SPB (23), respectively. Bar, 10 μm. (D) Inability of Hsk1 to complement sporulation defect of the $spo4$ mutant. Strain TN229 ($spo4$-$B4$) was transformed with plasmids in the following combinations: pREP41 plus pREP2, pREP41(spo4) plus pREP2, pREP41(hsk1) plus pREP2, or pREP41(hsk1) plus pREP2(HA-Him1). The transformants were sporulated on SSA medium for 2 days at 28°C. HA-tagged Him1 is functional, as described by Takeda et al. (63). Bar, 10 μm.

We next examined the precise arrest phenotype of a homozygous $spo4\Delta$ diploid strain during meiosis. Meiosis was induced by transferring a log-phase culture to nitrogen-free medium. $spo4\Delta$ cells proceeded through meiosis I with kinetics similar to those of the wild type. However, approximately 80% of $spo4\Delta$ cells arrested at the binucleate stage (Fig. 3B). Most of the $spo4\Delta$ cells (approximately 90%) arrested at the binucleate stage exhibited cytoplasmic microtubules (Fig. 3C), sug-

gesting that these cells were in the interkinesis between meiosis I and meiosis II. A small number of *spo4Δ* cells had pairs of chromatin regions insufficiently separated (Fig. 3C). At anaphase II, wild-type cells assembled spindles of interdigitated microtubules between SPBs (Fig. 3C). However, few intact spindles were seen in *spo4Δ* cells. Instead, these cells contained short fragments of microtubules associated with the SPB, indicating that the integrity of spindle microtubules would be impaired (Fig. 3C). We conclude that Spo4 is required for both entry into meiosis II and the progression through anaphase II.

S. pombe has another Cdc7-related kinase, Hsk1 (45). Hsk1 forms a complex with the Dbf4-related protein Dfp1/Him1 (6, 63). This complex is required for the initiation of DNA replication. To assess the level of functional relatedness between Spo4 and Hsk1, *hsk1+* was ectopically expressed under the control of the *nmt1* promoter in the *spo4* mutant. Such overexpression of *hsk1+* could not rescue the sporulation defect of the mutant (Fig. 3D). The same result was obtained when Hsk1 and Dfp1/Him1 were coexpressed in the *spo4* mutant (Fig. 3D). These observations suggest that Hsk1 cannot be substituted for Spo4.

Expression of Spo4. In budding yeast and other organisms, the abundance of either the transcript or protein of the Cdc7 kinase is constant throughout the cell cycle (61). We next explored whether the expression of *spo4+* was controlled in a similar manner. Synchronous meiosis was induced using JZ670 strains (*pat1-114*) (30), and the *spo4* mRNA levels were monitored by Northern blot analysis. *spo4* mRNA was barely detectable in vegetative cells, though it abruptly increased during meiosis. The timing of a burst of *spo4* transcription during meiosis was then more precisely determined with cells undergoing meiosis synchronously. Figure 4 shows that the induction of *spo4+* occurs with or just before the appearance of binucleate cells, that is, at the end of meiosis I. *spo4+* has a consensus sequence (GTAAACAAACA) named FLEX (1, 29) in the 5′ upstream region by which a meiosis-specific transcription factor, Mei4, recognizes its targets (Fig. 1C). In fact, transcription of *spo4+* was completely abolished in *mei4Δ* (Fig. 4A). In conclusion, the transcription of *spo4+* is induced during meiosis under the regulation of Mei4.

To identify the Spo4 protein, Spo4 was tagged with an HA epitope at the carboxyl terminus. This construct is fully functional, because it rescues meiotic defects of a *spo4Δ* mutant (data not shown). SDS-PAGE analysis showed that the Spo4-HA protein was not detectable in cell extract from vegetative cells, while it appeared as a 53-kDa polypeptide in meiotic-cell extract (Fig. 5A). Spo4-HA protein was most abundant in binucleate cells which completed meiosis I (Fig. 5B).

To address the question of whether protein kinase activity is a prerequisite for the meiotic function of Spo4, we introduced a series of amino acid substitutions, such as K95A, D182N, T264A, and T264E, according to the method of Ohtoshi et al. (54) (Fig. 2C). These are mutations at an ATP-binding site (K95A) and a conserved kinase domain (D182) (25). Threonine 264 (T264) might correspond to T167 of *S. pombe* Cdc2, which is phosphorylated by Cdk activation kinase (8). We expressed these mutant genes under the control of the *nmt1* promoter in a *spo4Δ* mutant. As Fig. 2C shows, none of these mutant *spo4* genes complement the sporulation defect of the
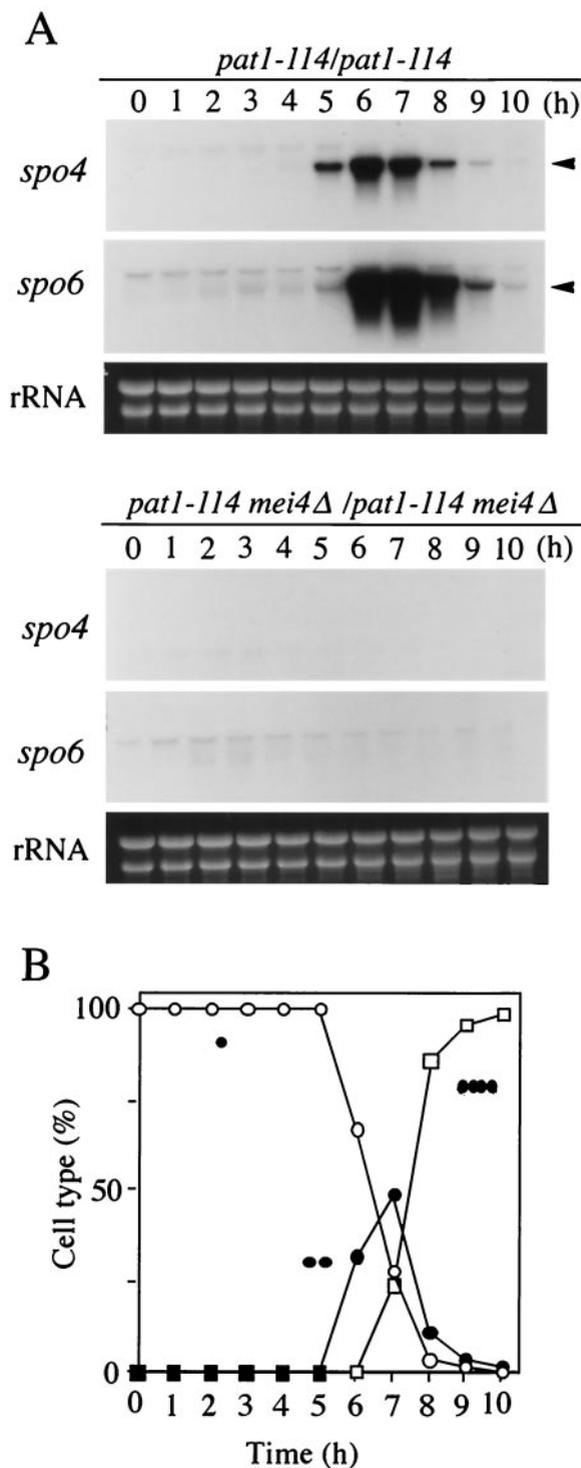

FIG. 4. (A) Transcription of the *spo4+* gene in *pat1*-driven synchronous meiosis (30). Meiosis of the diploid strains harboring homozygous *pat1-114*, JZ670 (*mei4+*), and AB4 (*mei4Δ*) was synchronous, and at intervals total RNA was prepared and analyzed by Northern blot hybridization as described in Materials and Methods. Arrowheads, *spo4* and *spo6* mRNAs. The approximate quantity of RNA was checked by staining the gels with ethidium bromide, which reveals rRNA. (B) Meiotic nuclear division of JZ670 (*mei4+*) was monitored by DAPI staining. Frequencies of mononucleate cells (single dot; open circles), binucleate cells (two dots; solid circles), and tetranucleate cells (four dots; open squares) were determined.
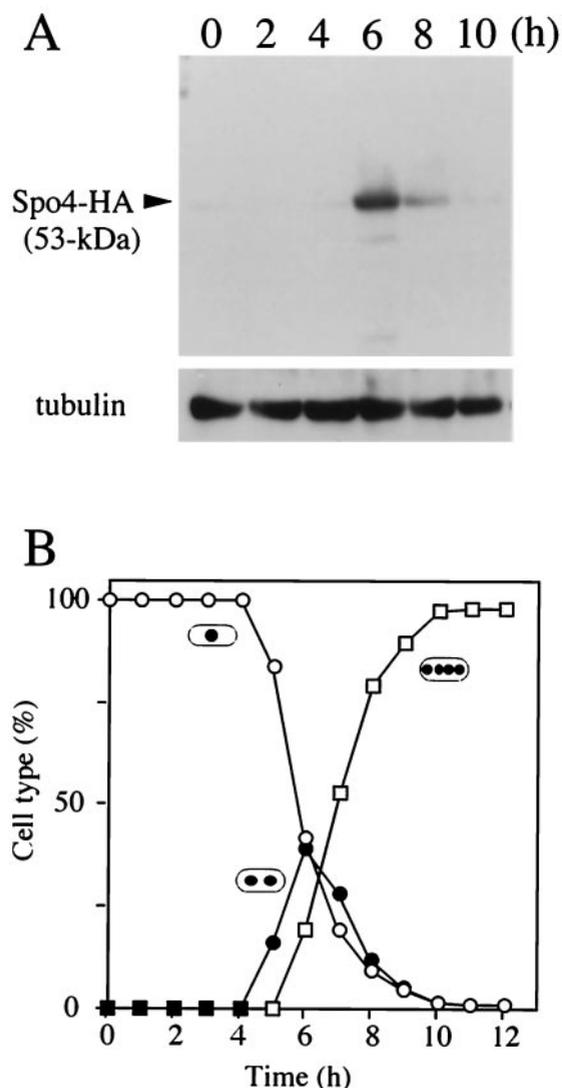
A

B

FIG. 5. Time course of expression of Spo4 during meiosis. (A) Cells homozygous for a gene replacement of Spo4-HA (TN188) were induced to undergo synchronous meiosis (30). Aliquots were removed every 2h, and protein extracts were subjected to immunoblot analysis with the mouse anti-HA antibody 12CA5, as well as with anti-α-tubulin antibody as the loading control. (B) Meiotic nuclear division was monitored by counting the number of nuclei. Frequencies of mononucleate cells (single dot; open circles), binucleate cells (two dots; closed circles), and tetranucleate cells (four dots; open squares) were determined.
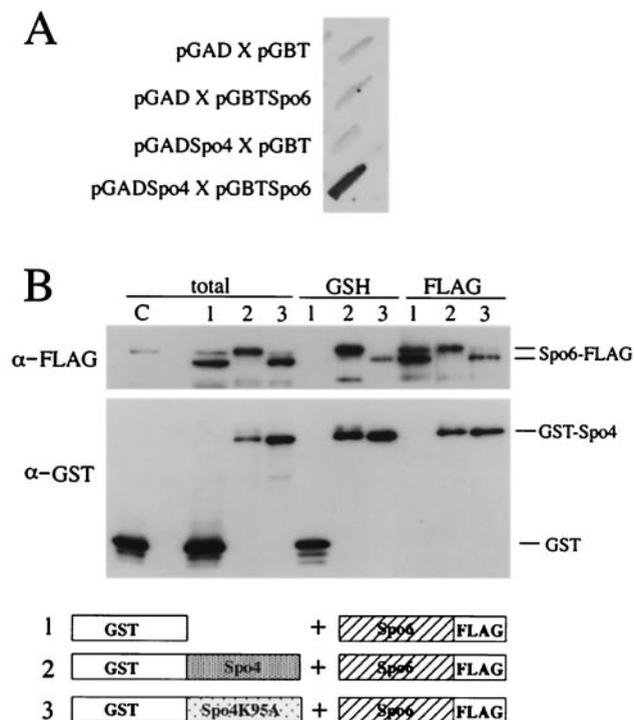
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FIG. 6. Spo4 interacts with Spo6. (A) Yeast two-hybrid analysis. The interaction between Spo4 and Spo6 is positive, as judged by the blue coloring of the host cells. The entire lengths of Spo4 and Spo6 were fused to a Gal4 activation domain and a Gal4 DNA binding domain, respectively. Both chimeric proteins were expressed in *S. cerevisiae* strain Y187. The intensity of the blue is reproduced in black and white. (B) Spo4 interacts with Spo6 in vivo. (Top) GST (lanes 1), GST-Spo4 (lanes 2), and GST-Spo4^{K95A} (lanes 3) were coexpressed with FLAG-tagged Spo6. As a control, only GST was expressed (lane C). Total cell lysates (total) and proteins bound to glutathione beads (GSH) or anti-FLAG M2 affinity gel (FLAG) were analyzed by immunoblotting using anti-GST and anti-FLAG antibodies (α-GST and α-FLAG, respectively). Note that nonspecific cross-reaction was observed near the band of Spo6-FLAG. (Bottom) A diagrammatic representation of the fusion proteins expressed in cells. The numbers correspond to lanes.

spo4Δ mutant. Similarly, overexpression of the HA-tagged mutant proteins could not rescue the defect, although the HA-tagged wild-type Spo4 suppressed the spo4 mutation (data not shown). As the abundance of the mutant proteins was roughly the same as that of the wild-type HA-Spo4, the inability to complement spo4Δ is not due to the reduction of their expression levels (Fig. 2D). These results suggest that the protein kinase activity is probably essential for the Spo4 function and that posttranslational regulation might be implicated in the activity.

Spo4 forms an active kinase complex with a Dbf4-like protein, Spo6. The activity of Cdc7 kinase is controlled by its regulatory subunit, Dbf4. The abundance of Dbf4 fluctuates during the cell cycle, peaking at S phase. Recently, we identified a sporulation-specific gene, spo6$^+$, which encodes a Dbf4-like protein in fission yeast (50). Therefore, it is possible that Spo4 is complexed with Spo6. spo6 mutants exhibit defective phenotypes in the second meiotic division and in sporulation similar to spo4 mutants, though the phenotype of spo4Δ was slightly more severe than that of spo6Δ (50) (Fig. 3B). Furthermore, the spo4 defect in the initiation of the second meiotic division was not exaggerated by the null mutation of spo6. Additionally, the transcriptional induction of spo6$^+$ coincided with that of spo4$^+$ and was regulated in a Mei4-dependent manner (29) (Fig. 4A). These results are consistent with the idea that Spo4 and Spo6 form an active complex to execute the function essential for meiosis. This possibility was further confirmed by a yeast two-hybrid assay (2). The entire lengths of Spo4 and Spo6 were fused to a Gal4 activation domain and a Gal4 DNA binding domain, respectively. Both chimeric pro-
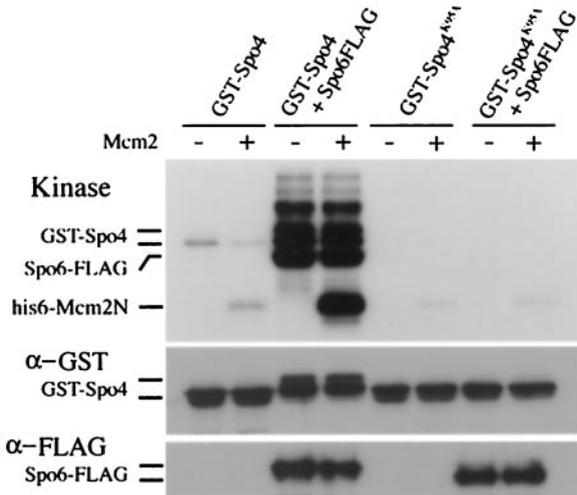
FIG. 7. Protein kinase assays of Spo4 protein. A homothallic strain, HA46-11B (spo6Δ), harboring either pREP41(GST-spo4) or pREP41 (GST-spo4 ^{K95A}), and TN29 (wild type) harboring pREP42(spo6-FLAG) and either pREP41(GST-spo4) or pREP41(GST-spo4 ^{K95A}) were cultured in MM for 12 h at 28°C. Extracts were prepared from each culture, proteins bound to glutathione beads were incubated with [γ-^{32}P]ATP in kinase assay buffer, and the products were examined by SDS-PAGE followed by autoradiography. The positions of Spo4, Spo6, and Mcm2 (added as a substrate) are shown. The Spo4 and Spo6 proteins were detected by immunoblotting using anti-GST and anti-FLAG antibodies, respectively. +, present; −, absent.

teins were expressed in *S. cerevisiae* strain Y187. Apparently, the assay indicated a positive interaction between the two proteins (Fig. 6A).

To assess the association of Spo4 and Spo6 in vivo, a chimeric protein of Spo4 fused to GST was coexpressed with FLAG-tagged Spo6 in *S. pombe*. GST-Spo4 was pulled down with glutathione beads, and copurification of Spo6 was verified by immunoblotting using anti-FLAG antibody. Spo6-FLAG was copurified with GST-Spo4 but not with unfused GST (Fig. 6B). Conversely, the immunoprecipitate obtained with anti-FLAG antibody contained GST-Spo4 (Fig. 6B). A kinase-negative Spo4 (GST-Spo4^{K95A}) was also able to associate with Spo6-FLAG. These results indicate that Spo4-FLAG and GST-Spo6 are physically associated and do not require the Spo4 kinase activity. It is noteworthy that the mobility of both GST-Spo4 and Spo6-FLAG on SDS-PAGE was retarded when wild-type Spo4 was expressed. Such mobility shifting was eliminated by treatment with phosphatase (data not shown), indicating that the shifted bands were hyperphosphorylated forms of Spo4 and Spo6. Such phosphorylated forms were not observed in cells expressing GST-Spo4^{K95A} (Fig. 6B).

Next we addressed the question of whether Spo4 has kinase activity in vitro. GST-Spo4 and GST-Spo4^{K95A} were expressed in either *spo6$^+$* or *spo6Δ* strains and partially purified using glutathione beads from cell extracts. The N-terminal region of *S. pombe* Mcm2 (Nda1/Cdc19) protein was used as a potential substrate (18, 48), because known Cdc7-related kinases phosphorylate Mcm2 in vitro (6, 44, 63). Autokinase and Mcm2 kinase activities were detected in the isolated GST-Spo4, whereas GST-Spo4^{K95A} abolished both activities (Fig. 7). The

control extract from the transformant bearing pREP41(GST) gave no activity (data not shown). In the presence of Spo6, both autokinase and Mcm2 kinase activities of Spo4 were markedly enhanced (Fig. 7). We conclude that Spo4 and Spo6 form an active kinase complex like Cdc7-Dbf4-related kinase complexes of other eukaryotes.

Spo4 localizes to the nucleus during early meiosis. Finally, we investigated the subcellular localization of Spo4-HA protein by indirect immunofluorescence microscopy. As expected, the Spo4-HA signal was not detectable in vegetative cells (data not shown). During meiosis, Spo4-HA preferentially localized in the nucleus (Fig. 8). The Spo4 signal in the nucleus, however, became less intense as cells proceeded through meiosis II. Noticeably, Spo6, a partner of Spo4, localized in the nucleus during meiosis (50). In *S. cerevisiae*, Dbf4 recruits Cdc7 to replication initiation complexes (13, 57, 64, 69). To clarify whether Spo6 determines the nuclear localization of Spo4, we observed the localization of Spo4 in the *spo6Δ* mutant. However, the nuclear localization of Spo4-HA was not affected by the *spo6* mutation (Fig. 8), suggesting that localization of Spo4-HA is independent of Spo6 function.

DISCUSSION

A serine/threonine protein kinase complex, Cdc7-Dbf4, plays a critical role in the initiation of S phase (4, 12, 13, 27, 28, 35, 43, 45, 57, 62, 63). The regulatory subunit, Dbf4, is a cyclin-like protein, in that its abundance periodically fluctuates, peaking at S phase, while Cdc7 levels remain constant throughout the cell cycle (10, 11, 17, 55). Dbf4 stimulates the
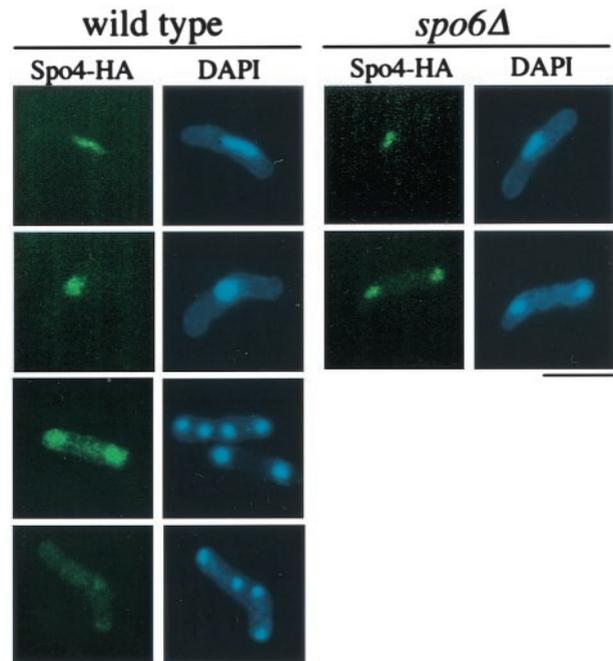

FIG. 8. Subcellular localization of Spo4-HA during meiosis. The homothallic haploid strains TN194 (wild type) and TN195 (spo6Δ) harboring chromosomally integrated Spo4-HA were cultured in SSL-N to induce meiosis. Spo4-HA was observed at different stages of meiosis after fixation using Rat anti-HA antibody 3F10. Bars, 10 μm.

kinase activity of the catalytic subunit, Cdc7 (32, 42). As Dbf4 interacts with the origin recognition complex, it may be possible that Dbf4 recruits Cdc7 to the chromosomal replication origins (13, 49, 57, 64). The Cdc7 kinase is characterized by a well-conserved primary structure and the presence of kinase insert regions, as well as by complex formation with the Dbf4-like protein. The Cdc7-Dbf4 kinase complex is evolutionarily conserved among eukaryotic organisms (34, 35, 37, 40, 43, 46, 59). The fission yeast *S. pombe* has a homologous kinase complex, composed of Hsk1 and Dfp1/Him1, which is also essential for the onset of DNA replication (6, 7, 45, 63). The present and previous studies revealed that Spo4-Spo6 is a novel Cdc7-related kinase complex. First, Spo4 protein has significant sequence similarity to known Cdc7 kinases. In addition, it contains three kinase insert sequences at positions identical to those of *S. cerevisiae* and other Cdc7-related kinases. Furthermore, Spo4 forms a complex with the Dbf4-like protein, Spo6, which markedly enhances the Spo4 kinase activity. This interaction is essential for its function. We conclude from these features that the fission yeast Spo4-Spo6 complex is a novel Cdc7-Dbf4 kinase.

In spite of these common features, Spo4-Spo6 kinase has some unique characteristics. Spo4 did not have the C-terminal tail rich in acidic amino acids found in budding yeast Cdc7 and fission yeast Hsk1. This C-terminal tail is essential for the functions of the Cdc7 kinase (54). Two-hybrid assays indicated that Dbf4 protein interacted with the C-terminal acidic tail of Cdc7 protein in budding yeast (54). However, human and *Xenopus* Cdc7 proteins did not contain such C-terminal tails. Spo4 might interact with Spo6 in a manner analogous to human and *Xenopus* Cdc7 proteins.

The kinase activity of some conventional Cdc7 proteins is regulated by association with Dbf4, whose level is cell cycle regulated. Spo4 kinase activity was markedly enhanced by Spo6. Therefore, we conclude that the catalytic subunit, Spo4, is controlled by its associated subunit, Spo6. Cdc7 kinase has been suggested to be regulated by phosphorylation, though the kinase responsible is still unknown. The threonine residue at 264 in Spo4 is equivalent to the threonine residue at 167 of *S. pombe* Cdc2. Phosphorylation of the corresponding residue of budding yeast Cdc7 is required to activate the kinase (8). Both Spo4^{T264A} and Spo4^{T264E} mutant proteins turned out to be nonfunctional, suggesting that phosphorylation of this threonine residue is necessary for the function. The expression of known Cdc7-related kinases is not regulated throughout the cell cycle. In contrast, *spo4*$^+$ was transcriptionally regulated; it is barely transcribed in vegetative cells but is induced under the control of a meiosis-specific transcription factor, Mei4, during meiosis. Interestingly, the expression of *spo6*$^+$ is also regulated by Mei4. Thus, both *spo4*$^+$ and *spo6*$^+$ are coordinately expressed by the same transcription mechanism. This is in contrast to other eukaryotic members of the Cdc7-Dbf4 kinase family.

Cdc7-related proteins are known to localize to the nucleus (34, 59, 72). Likewise, Spo4 was detected in nuclei of meiotic cells, relatively abundantly in mono- and binucleate cells and less so in tetranucleate cells. This result was consistent with the fact that Spo4 has an essential function in the initiation and progression of meiosis II. In *S. cerevisiae*, as well as activating Cdc7, Dbf4 recruits Cdc7 to the replication initiation complex.

Spo6 has a putative nuclear localization signal (50), in contrast to Spo4, which has no such signal. However, localization of Spo4 to the nucleus does not depend on Spo6. Of course, we cannot exclude the possibility that Spo6 recruits Spo4 to a specific site within the nucleus.

All known Cdc7-related kinases are involved in the initiation of DNA replication. However, we found no obvious defects in DNA replication in the *spo4* mutant. What is the cellular function of the Spo4-Spo6 kinase complex? Most of the *spo4* mutant cells arrest at the interkinetic stage between meiosis I and II. A small fraction of the *spo4* cells that entered into meiosis II, however, exhibited poorly separated sister chromatids with fragmented spindles. These results indicate that *spo4*$^+$ is indispensable for initiation of the second meiotic division and probably for the maintenance of spindle integrity during meiosis II. Schild and Byers (60) reported that the budding yeast *CDC7* gene is also required for meiosis. *cdc7* mutants arrest at a pachytene stage of meiosis I, and the genetic recombination is severely impaired. Although both the *S. cerevisiae cdc7* mutant and *S. pombe spo4* mutant show meiotic defects, their phenotypes are totally different. Physiological substrates of Cdc7 kinase for meiotic function have not been identified. Like other Cdc7 kinases, Spo4 efficiently phosphorylates Mcm2 in vitro. Phosphorylation of Mcm2 by the Cdc7 kinases, including Hsk1, may regulate the essential function of the MCM complex in initiation of DNA replication. It is unlikely that in vivo phosphorylation of Mcm2, if it occurs, plays a physiological role in meiosis. Identification of the in vivo substrate of Spo4 is necessary for understanding the function of Spo4-Spo6 kinase in *S. pombe*.

In conclusion, *S. pombe* has at least two Cdc7-Dbf4 kinase complexes. One is Hsk1-Dfp1, and the other is Spo4-Spo6. Despite the sequence similarity, swapping of the partner results in loss of function. The Hsk1-Dfp1 complex has a cellular function very similar to those of other known Cdc7-related kinases, and its involvement in mitotic nuclear division has not been reported. In contrast, Spo4-Spo6 has a function different from those of any reported Cdc7-related kinases. This nonconventional type of Cdc7 homologue may expand the concept of the biological role of this family of kinases. It reminds us of the case of the cyclin-dependent kinases (Cdks). Cdk was first isolated as a cell cycle regulator. Recently a novel type of Cdk-related protein kinase, Pho85, was reported not to be involved in cell cycle regulation (38). To date, the fission yeast *S. pombe* is the only organism known to have two sets of Cdc7 kinase complexes, each with a different biological role. We could not find a second gene encoding Cdc7 and Dbf4 proteins in genome sequence databases of model organisms such as *S. cerevisiae* and *Caenorhabditis elegans*. Further progress in genome sequencing projects for other eukaryotes may elucidate organisms which contain multiple Cdc7 kinase complexes, like *S. pombe*.

REFERENCES

1. **Abe, H., and C. Shimoda.** 2000. Autoregulated expression of *Schizosaccharomyces pombe* meiosis-specific transcription factor Mei4 and a genome-wide search for its target genes. Genetics **154:**1497–1508.

2. **Bai, C., and S. J. Elledge.** 1996. Gene identification using the yeast two-hybrid system. Methods Enzymol. **273:**331–347.

3. **Beach, D., and P. Nurse.** 1981. High-transformation of the fission yeast *Schizosaccharomyces pombe*. Nature **290:**140–142.

4. **Bousset, K., and J. F. Diffley.** 1998. The Cdc7 protein kinase is required for origin firing during S phase. Genes Dev. **12:**480–490.

5. **Bresch, C., G. Muller, and R. Egel.** 1968. Genes involved in meiosis and sporulation of a yeast. Mol. Gen. Genet. **102:**301–306.

6. **Brown, G. W., and T. J. Kelly.** 1998. Purification of Hsk1, a minichromosome maintenance protein kinase from fission yeast. J. Biol. Chem. **273:**22083–22090.

7. **Brown, G. W., and T. J. Kelly.** 1999. Cell cycle regulation of Dfp1, an activator of the Hsk1 protein kinase. Proc. Natl. Acad. Sci. USA **96:**8443–8448.

8. **Buck, V., A. White, and J. Rosamond.** 1991. *CDC7* protein kinase activity is required for mitosis and meiosis in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **227:**452–457.

9. **Byers, B.** 1981. Cytology of the yeast life cycle, p. 59–96. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces cerevisiae*: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

10. **Chapman, J. W., and L. H. Johnston.** 1989. The yeast gene, *DBF4*, essential for entry into S phase, is cell cycle regulated. Exp. Cell Res. **180:**419–428.

11. **Cheng, L., T. Collyer, and C. F. Hardy.** 1999. Cell cycle regulation of DNA replication initiator factor Dbf4p. Mol. Cell. Biol **19:**4270–4278.

12. **Donaldson, A. D., W. L. Fangman, and B. J. Brewer.** 1998. Cdc7 is required throughout the yeast S phase to activate replication origins. Genes Dev. **12:**491–501.

13. **Dowell, S. J., P. Romanowski, and J. F. Diffley.** 1994. Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. Science **265:**1243–1246.

14. **Egel, R.** 1971. Physiological aspects of conjugation in fission yeast. Planta **98:**89–96.

15. **Egel, R., and M. Egel-Mitani.** 1974. Premeiotic DNA synthesis in fission yeast. Exp. Cell Res. **88:**127–134.

16. **Egel, R.** 1989. Mating-type genes, meiosis and sporulation, p. 31–73. *In* A. Nasim, P. Young, and B. F. Johnson (ed.), Molecular biology of the fission yeast. Academic Press, San Diego, Calif.

17. **Ferreira, M. F., C. Santocanale, L. S. Drury, and J. F. Diffley.** 2000. Dbf4p, an essential S phase-promoting factor, is targeted for degradation by the anaphase-promoting complex. Mol. Cell. Biol. **20:**242–248.

18. **Forsburg, S. L., and P. Nurse.** 1994. The fission yeast *cdc19+* gene encodes a member of the MCM family of replication proteins. J. Cell Sci. **107:**2779–2788.

19. **Forsburg, S. L., and D. A. Sherman.** 1997. General purpose tagging vectors for fission yeast. Gene **191:**191–195.

20. **Furuno, N., M. Nishizawa, K. Okazaki, H. Tanaka, J. Iwashita, N. Nakajo, Y. Ogawa, and N. Sagata.** 1994. Suppression of DNA replication via Mos function during meiotic divisions in *Xenopus* oocytes. EMBO J. **13:**2399–2410.

21. **Grimm, C., J. Kohli, J. Murray, and K. Maundrell.** 1988. Genetic engineering of *Schizosaccharomyces pombe*: a system for gene disruption and replacement using the *ura4* gene as a selectable marker. Mol. Gen. Genet. **215:**81–86.

22. **Gutz, H., H. Heslot, U. Leupold, and N. Loprieno.** 1974. *Schizosaccharomyces pombe*, p. 395–446. *In* R. C. King (ed.), Handbook of genetics. Plenum Press, New York, N.Y.

23. **Hagan, I., and M. Yanagida.** 1995. The product of the spindle formation gene *sad1+* associates with the fission yeast spindle pole body and is essential for viability. J. Cell Biol. **129:**1033–1047.

24. **Hagan, I. M., and J. S. Hyams.** 1988. The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe*. J. Cell Sci. **89:**343–357.

25. **Hanks, S. K., A. M. Quinn, and T. Hunter.** 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241:**42–52.

26. **Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge.** 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell **75:**805–816.

27. **Hartwell, L.** 1973. Genetic control of the cell division cycle in yeast: V. Genetic analysis of *cdc* mutants. Genetics **74:**267–286.

28. **Hartwell, L.** 1973. Three additional genes required for deoxynucleic acid synthesis in *Saccharomyces cerevisiae*. J. Bacteriol. **115:**966–974.

29. **Horie, S., Y. Watanabe, K. Tanaka, S. Nishiwaki, H. Fujioka, H. Abe, M. Yamamoto, and C. Shimoda.** 1998. The *Schizosaccharomyces pombe mei4+*

30. gene encodes a meiosis-specific transcription factor containing a forkhead DNA-binding domain. Mol. Cell. Biol. **18:**2118–2129.

31. **Iino, Y., Y. Hiramine, and M. Yamamoto.** 1995. The role of *cdc2* and other genes in meiosis in *Schizosaccharomyces pombe*. Genetics **140:**1235–1245.

31. **Ikemoto, S., T. Nakamura, M. Kubo, and C. Shimoda.** 2000. *S. pombe* sporulation-specific coiled-coil protein Spo15p is localized to the spindle pole body and essential for its modification. J. Cell Sci. **113:**545–554.

32. **Jackson, A. L., P. M. Pahl, K. Harrison, J. Rosamond, and R. A. Sclafani.** 1993. Cell cycle regulation of the yeast Cdc7 protein kinase by association with the Dbf4 protein. Mol. Cell. Biol. **13:**2899–2908.

33. **Jensen, R., G. F. Sprague, Jr., and I. Herskowitz.** 1983. Regulation of yeast mating-type interconversion: feedback control of *HO* gene expression by the mating-type locus. Proc. Natl. Acad. Sci. USA **80:**3035–3039.

34. **Jiang, W., and T. Hunter.** 1997. Identification and characterization of a human protein kinase related to budding yeast Cdc7p. Proc. Natl. Acad. Sci. USA **94:**14320–14325.

35. **Jiang, W., D. McDonald, T. J. Hope, and T. Hunter.** 1999. Mammalian Cdc7-Dbf4 protein kinase complex is essential for initiation of DNA replication. EMBO J. **18:**5703–5713.

36. **Johnston, L. H., and A. P. Thomas.** 1982. A further two mutants defective in initiation of the S phase in the yeast *Saccharomyces cerevisiae*. Mol. Gen. Genet. **186:**445–448.

37. **Johnston, L. H., H. Masai, and A. Sugino.** 1999. First the CDKs, now the DDKs. Trends Cell Biol. **9:**249–252.

38. **Kaffman, A., I. Herskowitz, R. Tjian, and E. K. O'Shea.** 1994. Phosphorylation of the transcription factor *PHO4* by a cyclin-CDK complex, *PHO80-PHO85*. Science **263:**1153–1156.

39. **Kearns, B. G., J. G. Alb, Jr., and V. Bankaitis.** 1998. Phosphatidylinositol transfer proteins: the long and winding road to physiological function. Trends Cell Biol. **8:**276–282.

40. **Kim, J. M., N. Sato, M. Yamada, K. Arai, and H. Masai.** 1998. Growth regulation of the expression of mouse cDNA and gene encoding a serine/threonine kinase related to *Saccharomyces cerevisiae CDC7* essential for G1/S transition. Structure, chromosomal localization, and expression of mouse gene for *S. cerevisiae* Cdc7-related kinase. J. Biol. Chem. **273:**23248–23257.

41. **Kishida, M., and C. Shimoda.** 1986. Genetic mapping of eleven *spo* genes essential for ascospore formation in the fission yeast *Schizosaccharomyces pombe*. Curr. Genet. **10:**443–447.

42. **Kitada, K., L. H. Johnston, T. Sugino, and A. Sugino.** 1992. Temperature-sensitive *cdc7* mutations of *Saccharomyces cerevisiae* are suppressed by the *DBF4* gene, which is required for the G1/S cell cycle transition. Genetics **131:**21–29.

43. **Kumagai, H., N. Sato, M. Yamada, D. Mahony, W. Seghezzi, E. Lees, K. Arai, and H. Masai.** 1999. A novel growth- and cell cycle-regulated protein, ASK, activates human Cdc7-related kinase and is essential for G1/S transition in mammalian cells. Mol. Cell. Biol. **19:**5083–5095.

44. **Lei, M., Y. Kawasaki, M. R. Young, M. Kihara, A. Sugino, and B. K. Tye.** 1997. Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. Genes Dev. **11:**3365–3374.

45. **Masai, H., T. Miyake, and K. Arai.** 1995. *hsk1+*, a *Schizosaccharomyces pombe* gene related to *Saccharomyces cerevisiae CDC7*, is required for chromosomal replication. EMBO J. **14:**3094–3104.

46. **Masai, H., E. Matsui, Z. You, Y. Ishimi, K. Tamai, and K. Arai.** 2000. Human Cdc7-related kinase complex. In vitro phosphorylation of MCM by concerted actions of Cdks and Cdc7 and that of a criticial threonine residue of Cdc7 by Cdks. J. Biol. Chem. **275:**29042–29052.

47. **Maundrell, K.** 1993. Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene **123:**127–130.

48. **Miyake, S., N. Okishio, I. Samejima, Y. Hiraoka, T. Toda, I. Saitoh, and M. Yanagida.** 1993. Fission yeast genes *nda1+* and *nda4+*, mutations of which lead to S-phase block, chromatin alteration and Ca²⁺ suppression, are members of the *CDC46/MCM2* family. Mol. Biol. Cell **4:**1003–1015.

49. **Moreno, S., A. Klar, and P. Nurse.** 1990. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. **194:**793–823.

50. **Nakamura, T., M. Kishida, and C. Shimoda.** 2000. The *Schizosaccharomyces pombe spo6+* gene encoding a nuclear protein with sequence similarity to budding yeast Dbf4 is required for meiotic second division and sporulation. Genes Cells **5:**463–479.

51. **Nakamura, T., M. Nakamura-Kubo, A. Hirata, and C. Shimoda.** 2001. The *Schizosaccharomyces pombe spo3+* gene is required for assembly of the forespore membrane and genetically interacts with *psy1+* encoding syntaxin-like protein. Mol. Biol. Cell, in press.

52. **Nakase, Y., T. Nakamura, A. Hirata, S. M. Routt, H. B. Skinner, V. A. Bankaitis, and C. Shimoda.** 2001. The *Schizosaccharomyces pombe spo20+* gene encoding a homologue of *Saccharomyces cerevisiae* Sec14 plays an important role in forespore membrane formation. Mol. Biol. Cell **12:**901–917.

53. **Ohsumi, K., W. Sawada, and T. Kishimoto.** 1994. Meiosis-specific cell cycle regulation in maturing *Xenopus* oocytes. J. Cell Sci. **107:**3005–3013.

54. **Ohtoshi, A., T. Miyake, K. Arai, and H. Masai.** 1997. Analyses of *Saccharomyces cerevisiae* Cdc7 kinase point mutants: dominant-negative inhibition

of DNA replication on overexpression of kinase-negative Cdc7 proteins. Mol. Gen. Genet. **254:**562–570.

55. **Oshiro, G., J. C. Owens, Y. Shellman, R. A. Sclafani, and J. J. Li.** 1999. Cell cycle control of Cdc7p kinase activity through regulation of Dbf4p stability. Mol. Cell. Biol. **19:**4888–4896.

56. **Parisi, S., M. J. McKay, M. Molnar, M. A. Thompson, P. J. van der Spek, E. van Drunen-Schoenmaker, R. Kanaar, E. Lehmann, J. H. Hoeijmakers, and J. Kohli.** 1999. Rec8p, a meiotic recombination and sister chromatid cohesion phosphoprotein of the Rad21p family conserved from fission yeast to humans. Mol. Cell. Biol. **19:**3515–3528.

57. **Pasero, P., B. P. Duncker, E. Schwob, and S. M. Gasser.** 1999. A role for the Cdc7 kinase regulatory subunit Dbf4p in the formation of initiation-competent origins of replication. Genes Dev. **13:**2159–2176.

58. **Patterson, M., R. A. Sclafani, W. L. Fangman, and J. Rosamond.** 1986. Molecular characterization of cell cycle gene CDC7 from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6:**1590–1598.

59. **Sato, N., K. Arai, and H. Masai.** 1997. Human and *Xenopus* cDNAs encoding budding yeast Cdc7-related kinases: in vitro phosphorylation of MCM subunits by a putative human homologue of Cdc7. EMBO J. **16:**4340–4351.

60. **Schild, D., and B. Byers.** 1978. Meiotic effects of DNA-defective cell division cycle mutations of *Saccharomyces cerevisiae*. Chromosoma **70:**109–130.

61. **Sclafani, R. A., M. Patterson, J. Rosamond, and W. L. Fangman.** 1988. Differential regulation of the yeast *CDC7* gene during mitosis and meiosis. Mol. Cell. Biol. **8:**293–300.

62. **Sclafani, R. A.** 2000. Cdc7p-Dbf4p becomes famous in the cell cycle. J. Cell Sci. **113:**2111–2117.

63. **Takeda, T., K. Ogino, E. Matsui, M. K. Cho, H. Kumagai, T. Miyake, K. Arai, and H. Masai.** 1999. A fission yeast gene, *him1*$^+$/*dfp1*$^+$, encoding a regulatory subunit for Hsk1 kinase, plays essential roles in S-phase initiation as well as in S-phase checkpoint control and recovery from DNA damage. Mol. Cell. Biol. **19:**5535–5547.

64. **Tanaka, T., and K. Nasmyth.** 1998. Association of RPA with chromosomal replication origins requires an Mcm protein, and is regulated by Rad53, and cyclin- and Dbf4-dependent kinases. EMBO J. **17:**5182–5191.

65. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77:**5201–5205.

66. **Watanabe, Y., Y. Lino, K. Furuhata, C. Shimoda, and M. Yamamoto.** 1988. The *S. pombe mei2* gene encoding a crucial molecule for commitment to meiosis is under the regulation of cAMP. EMBO J. **7:**761–767.

67. **Watanabe, Y., S. Shinozaki-Yabana, Y. Chikashige, Y. Hiraoka, and M. Yamamoto.** 1997. Phosphorylation of RNA-binding protein controls cell cycle switch from mitotic to meiotic in fission yeast. Nature **386:**187–190.

68. **Watanabe, Y., and P. Nurse.** 1999. Cohesin Rec8 is required for reductional chromosome segregation at meiosis. Nature **400:**461–464.

69. **Weinreich, M., and B. Stillman.** 1999. Cdc7p-Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the RAD53 checkpoint pathway. EMBO J. **18:**5334–5346.

70. **Woods, A., T. Sherwin, R. Sasse, T. H. MacRae, A. J. Baines, and K. Gull.** 1989. Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. J. Cell Sci. **93:**491–500.

71. **Yamamoto, M., Y. Imai, and Y. Watanabe.** 1997. Mating and sporulation in *Schizosaccharomyces pombe*, p. 1037–1106. *In* J. R. Pringle, J. B. Broach, and E. W. Jones (ed.), Molecular and cellular biology of the yeast *Saccharomyces*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

72. **Yoon, H. J., and J. L. Campbell.** 1991. The *CDC7* protein of Saccharomyces cerevisiae is a phosphoprotein that contains protein kinase activity. Proc. Natl. Acad. Sci. USA **88:**3574–3578.