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Identification and characterization of genes induced during sexual differentiation in *Schizosaccharomyces pombe*

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Abstract. Five cDNA clones, harboring genetic messages preferentially expressed during the sexual differentiation process, were isolated from a cDNA library of *Schizosaccharomyces pombe* by subtractive screening. Transcription of the corresponding genes, termed *isp3, 4, 5, 6, and 7,* was dependent on nitrogen starvation and their induction occurred at several stages of spore formation. Analysis of the cDNA primary structures revealed a capacity for the coding of polypeptides of 19.2 kDa, 88.3 kDa, 60.1 kDa, 49.7 kDa, and 43.8 kDa, respectively. The translated amino-acid sequences of *isp5* and *isp6* were found to show significant similarities to those of amino-acid permeases and proteinase B of *Saccharomyces cerevisiae,* respectively. Disruption of *isp6* arrested the cell cycle prior to conjugation and caused a drastic blocking effect on spore formation.

Key words: *Schizosaccharomyces pombe* – Sexual differentiation – Stage-specific genes – Subtractive screening

Introduction

In yeasts, nitrogen starvation triggers sexual differentiation, i.e., mating of haploid cells of different mating types, meiosis and spore formation (Egel 1971; Esposito and Klapholz 1981). So-far, a number of mutants which alter phenotypes at various stages of this process have been isolated (Baker et al. 1976; Egel et al. 1990). Intensive studies of such mutants using the combined techniques of genetics and molecular biology have given us a partial outline of the underlying genetical events (Egel 1989; Malone 1990). However, to understand the whole process in detail, other participating genes require identification and analysis in terms of their function and regulation.

In an attempt to complement the conventional methodology in genetics, differential screening has been adopted to identify unknown genes directly from cDNA or genomic libraries (St. John and Davis 1979; Cochran et al. 1984). By using this method, novel genes expressed preferentially during sporogenesis have been isolated and characterized in the budding yeast, *Saccharomyces cerevisiae* (Clancy et al. 1983; Percival-Smith and Segall 1984). Here, we describe identification and characterization of genes preferentially transcribed during sexual differentiation in the fission yeast, *Schizosaccharomyces pombe*. In this study, we applied a similar but more sensitive technique, subtractive hybridization, to isolate cDNA clones harboring genetic messages abundantly expressed during the sexual differentiation process. Stage-specific cDNAs thus obtained were then subjected to primary structural analysis. Preliminary functional analysis of the corresponding genes was also carried out by gene-disruption and complementation experiments.

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Materials and methods

Strains and culture conditions. The strains of *S. pombe* used in this study are listed in Table I. For vegetative growth of the yeast, YPD and SD media were used (Iino and Yamamoto 1985). For induction of mating and sporulation, cells were cultured in SPA medium (Gutz et al. 1974). To synchronize the sporulation process, the method of Egel and Egel-Mitani (1974) was modified. Cells grown in nitro-

Table 1. *Schizosaccharomyces pombe* strains used

Strain	Genotype	
JY274	h^+/h^-	*his2/+ ade6-M210/ade6-M216*
JY275	h^+/h^+	*his2/+ ade6-M210/ade6-M216*
JY765	h^+/h^-	*leu1/leu1 ura4-D18 /ura4-D18 ade6-M210 /ade6-M216*
JY776	h^+/h^-	*leu1/leu1 ura4-D18/ura4-D18 ade6-M210/ ade6-M216 mei2::ura4*$^+$*/mei2::ura4*$^+$
JY800	h^{90}	*leu1 ura4-D18 ade6-M216*
SS003	h^{90}	*leu1 ura4-D18 ade6-M216 isp3::ura4*$^+$
SS004	h^{90}	*leu1 ura4-D18 ade6-M216 isp4::ura4*$^+$
SS005	h^{90}	*leu1 ura4-D18 ade6-M216 isp5::ura4*$^+$
SS006	h^{90}	*leu1 ura4-D18 ade6-M216 isp6::ura4*$^+$
SS007	h^{90}	*leu1 ura4-D18 ade6-M216 isp7::ura4*$^+$

gen-enriched medium (SSL+N) were transferred into nitrogen-free synthetic medium without glucose (SSL–N–C). After incubation for 2 h, glucose was added to a final concentration of 1%, and additional incubation carried out.

Genetic methods. Crossing, sporulation, transformation, and gene disruption were carried out by standard genetic techniques (Gutz et al. 1974; Moreno et al. 1991). Disruption mutants were constructed by introducing a 1.8-kb DNA fragment containing the *ura4* gene (Grimm et al. 1988) into two recipient strains. For examining the viability of the disruptants, JY765 was used to obtain haploid mutants after meiosis. JY800 was employed to obtain homothallic mutants in a single step, and the resulting strains, SS003, SS004, SS005, SS006, and SS007, were subjected to spore-formation testing. Insertion of the marker fragment into the target genes was confirmed by genomic Southern analysis.

Isolation of RNA. Total RNA was isolated from *S. pombe* cells as described by Sherman et al. (1986). Poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography (Sambrook et al. 1990).

Construction of a cDNA library. cDNA was synthesized using a ZAP-cDNA synthesis kit (Stratagene). Poly(A)$^+$ RNAs, isolated from JY274 which had been incubated in SSL–N for 4, 6 and 8 h, were mixed and used as the template. Following *Eco*RI adaptor ligation to the 5′ terminus and *Xho*I digestion, the cDNA was inserted into the *Eco*RI-*Xho*I site of lambda Zap (Stratagene).

Subtractive hybridization. ^{32}P-labelled cDNA was synthesized using 1 µg of poly(A)$^+$ RNA mixture purified from JY274 which had been incubated in SSL–N for 4, 6 and 8 h, as for the template, and using oligo(dT) as the primer. Ten micrograms of poly(A)$^+$ RNAs, prepared from JY776 which had been incubated for 4, 6, and 8 h in SSL–N, and from JY274 propagated in SSL+N, were biotinylated with photobiotine acetate and used as drivers for subtractive hybridization. ^{32}P-labelled cDNA from JY 274 and either one of the biotinylated poly(A)$^+$ RNAs mentioned above were mixed and hybridized at 68 °C for 48 h. Streptavidin was added to the hybridization mixture, and the resulting streptavidin-biotinylated poly(A)$^+$ RNA complex was removed by phenol-chloroform extraction. The cDNA in the aqueous phase was further purified by rehybridization against the same driver RNA, and the ^{32}P-labelled single-stranded cDNA thus obtained was used as a probe for screening of the cDNA library. Plaque hybridization was carried out according to the standard procedure (Benton and Davis 1977).

Northern analysis. Poly(A)$^+$ RNA isolated from 2×10^8 cells (approximately 2.5 µg) was denatured in 50% formamide and 6% formaldehyde at 60 °C, and separated on 1.1% agarose gels containing 6.7% formaldehyde. Transfer to nylon membranes (Hybond-N, Amersham) and hybridization were carried out according to a laboratory manual (Sambrook et al. 1989).

Pulsed-field gel electrophoresis. DNA samples were prepared according to Fan et al. (1988). Pulsed-field gel electrophoresis was carried out using a CHEF Mapper (BioRad). After the separation, DNA was transferred onto nylon membranes (Hybond-N, Amersham) (Southern 1975) and hybridization tests were carried out.

DNA sequencing and primary structure analysis. DNA sequences were determined according to the method of Sanger et al. (1977). The sequencing reaction was performed using a DyePrimer *Taq* cycle sequencing kit (Applied Biosystems) with Catalyst 800 (Applied Biosystems) and manually with a Dye deoxyterminator cycle sequencing kit (Applied Biosystems). Sequence analyses were performed on an ABI automated sequencer (373A, Applied Biosystems). For primary structure analyses of DNA and proteins, the GCG package (Devereux et al. 1984) was used.

Microscopic observation of the spore-formation process. After incubation in SPA for 24 h, cells were examined by Nomarski differential-interference-contrast microscopy for formation of spores. Cells were also observed with a fluorescent microscope after being stained with 1 µg/ml of DAPI (4,6-diamidino-2-phenylindole).

Results

Isolation of cDNA clones preferentially expressed during the sexual differentiation process

To identify genetic messages expressed during sexual differentiation in the fission yeast, *S. pombe*, a cDNA library derived from meiotic cells was screened with two kinds of specific probes, M and N, prepared as follows. Poly(A)$^+$ RNAs were purified from JY274 incubated in the sporulation medium for 4, 6 and 8 h. Using a mixture of the above poly(A)$^+$ RNAs as a template, cDNA was synthesized in the presence of ^{32}P-labelled deoxyribonucleotide. The labelled cDNA was subjected to hybridization with an excess amount of poly(A)$^+$ RNAs from the *mei2*-deficient cells and the somatic JY274 cells, followed by subtraction of annealed molecules, producing single-stranded M- and N-probes, respectively. *mei2* is expressed at a very early stage of prophase and its gene function is essential for the initiation of meiosis (Shimoda et al. 1987; Watanabe et al. 1988). The M-probe, therefore, should contain segments of cDNA corresponding to genes whose transcription is dependent on *mei2* gene function. The N-probe, on the other hand, should correspond to genes transcribed under nitrogen starvation conditions during meiosis and sporogenesis.

By screening approximately 50 000 plaques from the meiotic cDNA library, 50 clones with the M-probe and 30 clones with the N-probe showing positive signals were obtained. They were classified according to cross-hybridization tests into five groups and the clones containing the longest cDNA fragments were selected from each group. These clones were designated as pMI and the corresponding genes as *isp* (genes *i*nduced during *s*porogenesis in *S. pombe*). pMI3 and pMI4, which hybridized to both M- and N-probes, and pMI5, 6 and 7, specific for the N-probe, were thus obtained and subjected to further analyses.

Transcription of the isp *genes*

To examine the time course of transcription of the *isp* genes during sexual differentiation, poly(A)$^+$ RNA was extracted from a culture of sporogenous strain JY274 every 2 h after the induction of meiosis, and hybridized with corresponding cDNA fragments. As shown in Fig. 1, stage-specific transcription of *isp* genes was evident since accumulation of transcripts was clearly observed when the cells were transferred into the sporulation media. Transcription of *isp3* began 8 h after induction. This corresponds to the first meiotic division under the conditions used in this study. The transcript of *isp4* also accumulated 4 h after induction when the cells were still involved in premeiotic cell division. The genes, *isp5*, *6* and *7*, on the other hand, showed a quick response, with transcripts gradually increasing 2 h after induction when the cells were transferred

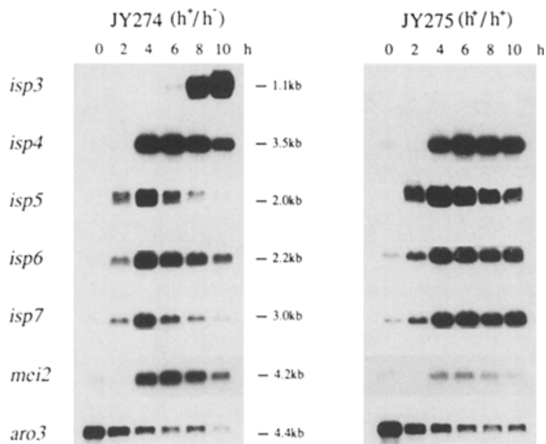

Fig. 1. Profiles of transcripts accumulated during sexual differentiation. Poly(A)$^{+}$RNAs were extracted from JY274 and JY275, incubated in the sporulation media for the indicated hours, and separated by agarose-gel electrophoresis. Northern hybridization was carried out with cDNA fragments, representing each gene indicated in the figure, as probes. DNA fragments containing the *aro3* gene (Nakanishi and Yamamoto 1984) and the *mei2* gene (Shimoda et al. 1987; Watanabe et al. 1988) were also used as control probes. Lengths of the respective transcripts are also shown

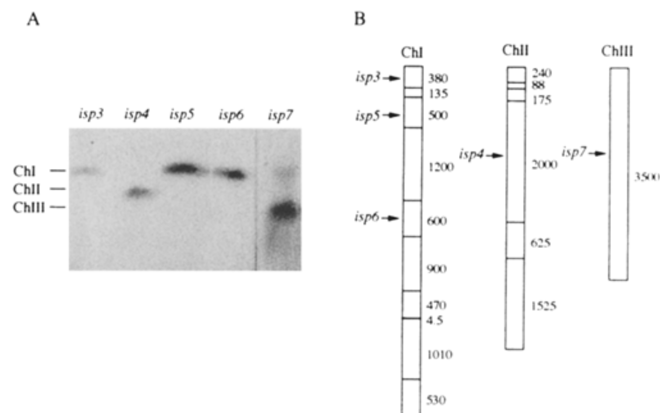

Fig. 2 A, B. Chromosome mapping of *isp* genes. **A** three chromosomes were separated by pulsed-field gel electrophoresis, and Southern-hybridization analysis was carried out using cDNA fragments, representing the respective genes, as probes. The chromosome number is indicated on the left. **B** *Not*I digests of genomic DNA were subjected to Southern-hybridization analysis (data not shown), and the fragments which hybridized to the respective probes are shown on a physical map (Smith and Cantor 1987; Fan et al. 1988)

to medium depleted with both glucose and nitrogen, and reaching their maximum at 4 h after induction.

When an asporogenous homozygous strain, JY275, was examined in place of JY274 (Fig. 1), different profiles were observed. pMI3 gave no detectable band, indicating dependency of transcription of the *isp3* gene on mating-type gene products. In contrast, *isp4*, *5*, *6*, and *7* exhibited similar profiles to those of JY274, though transcripts decreased more slowly as the process advanced.

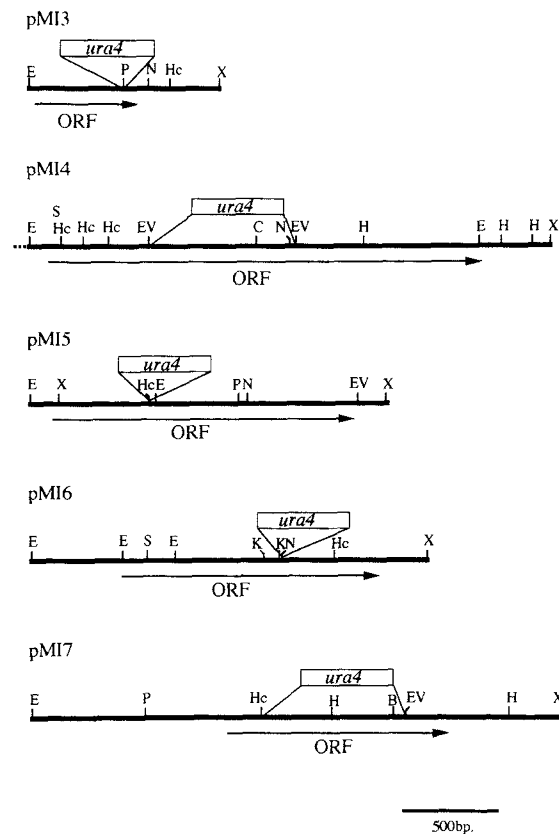

Fig. 3. Schematic representation of the primary structures of the cDNAs in pMI clones. Positions and direction of open reading frames (ORF) are shown by *arrows*. Poly-A tails are located inside of the *Xho*I sites at the right termini of the cDNAs. For gene disruption, a DNA fragment containing the *ura4* gene was inserted into the restriction sites shown at the top, as described in the Materials and methods. Restriction sites for *Eco*RI (*E*), *Xho*I (*X*), *Pst*I (*P*), *Sal*I (*S*), *Hin*dIII (*H*), *Kpn*I (*K*), *Nco*I (*N*), *Cla*I (*C*), *Bam*HI (*B*), *Eco*RV (*EV*), and *Hin*cII (*Hc*) are also indicated. The nucleotide sequence data appear in the DDBJ/EMBL/GenBank DNA databases under the accession numbers; pMI3 (D14060), pMI4 (D14061), pMI5 (D14062), pMI6 (D14063), and pMI7 (D14064)

Localization of isp *genes*

Three chromosomes of *S. pombe* were separated by pulsed-field gel electrophoresis, and Southern hybridization analysis was carried out using cDNA fragments of the respective pMI plasmids as probes. As shown in Fig. 2 A, pMI3, 5 and 6 hybridized to chromosome I, while pMI4 and pMI7 associated with chromosomes II and III, respectively. For more detailed mapping, a *Not*I digest of the genomic DNA was subjected to hybridization testing (data not shown). As a result, *isp* genes could be localized on the *Not*I physical map (Smith and Cantor 1987; Fan et al. 1988), as shown in Fig. 2 B.

Structural analysis of cDNAs

Comparing the lengths of the cDNA inserts and those of the corresponding mRNAs (Figs. 1 and 3), it was consid-

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Isp5   ---------- ---------- ---------- ---------- ----MPAMKR KKLDMESSRW FPKGETCFQR WYRSFLPPED GKPQKLKRTL TARHIQMIGI   56
Gap1   MSNTSSYEKN NPDNLKHNGI TIDSEFLTQE PITIPSNGSA VSIDETGSGS KWQDFKDSFK RVKPIEVDPN LSEAEKVAII TAQTPLKHHL KNRHLQMIAI  100
Hip1   MPRNPLKKEY WADVVDGFKP ATSPAFENEK ESTTFVTELT SKTDSAFPLS SKDSPGINQT TNDITSSDRF RRNEDTEQED INNTNLSGDL SVRHLLTLAV  100
Can1   --MTNSKEDA DIEEKHMYNE PVTTLFHDVE ASQTHHRRGS IPLKDEKSKE LYPLRSFPTR VNGEDTFSME DGIGDEDEGE VQNAEVKREL KQRHIGMIAL   98

Isp5   GGAIGTGVWV GSKNTLREGG AASVLICYSL VGSMVLMTVY SLGELAVAFP INGSFHTYGT RFIHPSWGFT LGWNYLASFL ATYPLELITA SICLQFW--I  154
Gap1   GGAIGTGLLV GSGTALRTGG -PSLLIGWGS TGTMIYAMVM ALGELAVIFP ISGGFTTYAT RFIDESFGYA NNFNYMLQWL VVLPLEIVAA SITVNFWGTD  199
Hip1   GGAIGTGLYV NTGAALSTGG PASLVIDWVI ISTCLFTVIN SLGELSAAFP VVGGFNVYSM RFIEPSFAFA VNLNYLAQWL VLLPLELVAA SITIKYW-ND  199
Can1   GGTIGTGLFI GLSTPLTNAG PVGALISYLF MGSLAYSVTQ SLGEMATFIP VTSSFTVFSQ RFLSPAFGAA NGYMYWFSWA ITFALELSVV GQVIQFW-TY  197

Isp5   NINSGIWITV FIALLCFVNM FGVRGYGEVE FFVSSLKVMA MVGFIICGIV IDCGGVRTDH RG--YIGATI FRK-NAF--- --IHGFHGFC SVFSTAAFSY  246
Gap1   PKYRDGFVAL FWLAIVIINM FGVKGYGEAE FVFSFIKVIT VVGFIILGII LNCGGGPTG- -G--YIGGKY WHDPGAFAGD TPGAKFKGVC SVFVTAAFSF  295
Hip1   KINSDAWVAI FYATIALANM LDVKSFGETE FVLSMIKILS IIGFTILGIV LSCGGGPHG- -G--YIGGKY WHDPGAFVGH SSGTQFKGLC SVFVTAAFTY  295
Can1   KVPLAAWISI FWVIITIMNL FPVKYYGEFE FWVASIKVLA IIGFLIYCFC MVCGAGVTGP VGFRYWRNPG AWGPGIISKD KNEGRFLGWV SSLINAAFTF  297

Isp5   AGTEYIGIAA SETKNPAKAF PKAVKQVFIR VSLFYILALF VVSLLISGRD ERLT-TLSA- --TAASPFIL ALMDAKIRGL PHVLNAVILI SVLTAANGIT  342
Gap1   AGSELVGLAA SESVEPRKSV PKAAKQVFWR ITLFYILSLL MVGLLVPYND KSLI-GASSV -DAAASPFVI AIKTHGIKGL PSVVNVVILI AVLSVGNSAI  393
Hip1   SGIEMTAVSA AESKNPRETI PKAAKRTFWL ITASYVTILT LIGCLVPSND PRLL-NGSSS VDAASSPLVI AIENGGIKGL PSLMNAIILI AVVSVANSAV  394
Can1   QGTELVGITA GEAANPRKSV PRAIKKVVFR ILTFYIGSLL FIGLLVPYND PKLTQSTSY- --VSTSPFII AIENSGTKVL PHIFNAVILT TIISAANSNI  394

Isp5   YTGSRTLHSM AEQGHAPKWF KYVDREGRPL LAMAFVLCFG ALGYICESAQ SDTVFDWLLS ISNLATLFVW LSINVSYIIY RLAFKKQGKS YDEVGYHSPF  442
Gap1   YACSRTMVAL AEQRFLPEIF SYVDRKGRPL VGIAVTSAFG LIAFVAASKK EGEVFNWLLA LSGLSSLFTW GGICICHIRF RKALAAQGRG LDELSFKSPT  493
Hip1   YACSRCMVAM AHIGNLPKFL NRVDKRGRPM NAILLTLFFG LLSFVAASDK QAEVFTWLSA LSGLSTIFCW MAINLSHIRF RQAMKVQERS LDELPFISQT  494
Can1   YVGSRILFGL SKNKLAPKFL SRTTKGGVPY IAVFVTAAFG ALAYMETSTG GDKVFEWLLN ITGVAGFFAW LFISISHIRF MQALKYRGIS RDELPFKAKL  494

Isp5   GIYGACYGA- FIIILVFITE FYVSIFPIGA SPDAGAFFQS YLCFPVVVIV FIAHALITR- -QKFRKLSEI DLDTGFSKYD RLEESDKGPM TAKSLAKSVL  539
Gap1   GVWGSYWGL- FMVIIMFIAQ FYVALFPVGD SPSAEGFFEA YLSFPLVMVM YIGHKIYKRN WKLFIPAEKM DIDTGRREVD LDLLKQEIAE EKAIMATKPR  592
Hip1   GVKGSWYGF- IVLFLVLIAS FWTFSVPIRR FRSQRRIIL- ---------- ---------- ---------- ---------- ---------- ----------  532
Can1   MPGLAYYAAT FMTIIIIIQG FTAFAPKFNG VSFAAAYISI FL-FLAVWIL F-QCIFRCR- --FIWKIGDV DIDSDRRDIE AIVWEDHEPK TFWDKFWNVV  589

Isp5   SFCV------   543
Gap1   WYRIWNFWC    601
Hip1   ----------   532
Can1   A---------   590
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Fig. 4. Comparison of amino-acid sequences of the *isp5* gene product and amino-acid permeases in *S. cerevisiae*. The entire sequences of the *isp5, GAP1* (Jauniaux and Grenson 1990), *HIP1* (Tanaka and Fink 1985), and *CAN1* (Hoffmann 1985) gene products are aligned. The positions showing amino-acid identity are indicated by *shadowing*

ered very probable that the respective cDNAs contain most of the coding regions. To speculate on the function of the gene products, the primary structure of the cDNA portions of the pMI clones was determined, and the sequence data obtained were subjected to computer analysis. A distinct open reading frame was observed in each clone (Fig. 3), and in-frame termination codons upstream of the assigned initiation codons were identified. The putative gene products of *isp3, 4, 5, 6,* and *7* were estimated as molecules of 19.2, 88.3, 60.1, 49.7, and 43.8 kDa, respectively (Figs. 4, 5 and 6).

The translated amino-acid sequence of Isp5 exhibits similarity to those of amino-acid permeases in *S. cerevisiae* . The relative identities with a general amino-acid permease, Gap1(Jauniaux and Grenson 1990), a histidine permease, Hip1 (Tanaka and Fink 1985), and an arginine permease, Can1(Hoffmann 1985), are 45.8%, 39.8%, and 34.6%, respectively (Fig. 4).

The putative product of *isp6* shows significant sequence similarity to proteinase B in *S. cerevisiae* (Moehle et al. 1987), alkaline proteinase in *Aspergillus oryzae* (Tatsumi et al. 1989), proteinase K in *Tritirachium album* (Jany et al. 1986), and Carlsberg subtilisin in *Bacillus subtilis* (Smith et al. 1978). Amino-acid identity between the regions spanning deduced mature polypeptides was found to reach 56.9%, 46.6%, 44.1%, and 37.6%, of the proteins mentioned above, respectively (Fig. 5). Homology between Isp6 and proteinase B in *S. cerevisiae* extends into prosequence regions. Three amino-acid residues known as the catalytic active site are conserved among the five proteinases .

Comparison of the nucleotide and deduced amino-acid sequences of Isp3, 4, and 7 with those in the databases did not show any sequence similarity (Fig. 6). One characteristic feature to be noted is the C-terminus of the *isp3* gene product, where short stretches of alanine residues are interrupted by threonine and proline (Fig. 6). A similar feature was reported in a cDNA clone, I3, specific to developing microsporocytes in *Brassica napus* (Roberts et al. 1991).

Disruption of isp *genes*

A DNA fragment containing the *ura4* gene was artificially inserted into the assigned coding regions of the *isp* genes (Fig. 3), and the effects of this on both mitotic and reproductive cell cycles assessed. When such mutants were propagated in YPD medium, no effect was seen on the vegetative growth of haploid and diploid cells. In sporulation medium, however, significant loss of ability to produce spores was detected in the *isp3* and *isp6* disruptants (Table 2). When SS003, a disruptant of *isp3*, was examined for spore forming ability, 45.5% of the cells formed spores, compared with 70.5% in the wild-type. The spores, once produced, maintained normal viability. When the DNA fragment containing the *ura4* gene was introduced into *isp6*, no spores were observed.

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Isp6   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------MRI    3
CEREVI MKLENTLFTL GALGSISAAL VIPNLENAAD HHELINKEDH HERPRKVEFT KDDDEEPSDS EDKEHGKFHK KGRKGQDKES PEFNGKRASG SHGSAHEGGK  100
ASPERG ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
PROK   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SUBTI  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

Isp6   PYSNLFSAAA GLALFASTAC AAPVMPATDS DIAHAGIRPE LDNAFYDSHG EAATPKHKPH AGPNAAPLLS ASNADTTGLD SHYIIVLQPD LSEQEFQAHT  103
CEREVI GMKPKHESSN DDDNDDKKKK PHHKGGCHEN KVEEKKMKGK KVKGKKHHEK TLEKGRHHNR LAPLVSTAQF NPDAISKIIP NRYIIVFKRG APQEEIDFHK  200
ASPERG ---------- ---------- ---------- ---------- ------MQSI KRTLLLLGAI LPAVLGAPVQ ETRRAAEKLP GKYIVTFKPG IDEAKIQEHT   54
PROK   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SUBTI  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

Isp6   NWV--SEMHQ MDIASQEDEY Y----DTS-D SNYMFGLKHV YDFGEDSFKG YSGQFSSNIV EQIRLHPHVI AVERDQVVSI KKLETQSGAP WGLARISHK-  195
CEREVI ENVQQAQLQS VENLSAEDAF FISTKDTSLS TSEAGGIQDS FNI-DNLFSG YIGYFTQEIV DLIRQNPLVD FVERDSIVEA TEFDTQNSAP WGLARISHRE  299
ASPERG TWA--TNIHQ RSLERRGAT- ---------G GDLPVGIERN YKI--NKFAA YAGSFDDATI EEIRKNEDVA YVEEDQIYYL DGLTTQKSAP WGLGSISHK-  139
PROK   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---AAQTNAP WGLARI----   13
SUBTI  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---AQTVPY-    6
                                   *                                   *
Isp6   SVKYDDIGKY VYDSSAGDNI TAYVVDTGVS IHHVEFEGRA SWGATIPSGD VDEDNNGHGT HVAGTIAS-- ---RAYGVAK KAEIVAVKVL RSSGSGTMAD  290
CEREVI RLNLGSFNKY LYDDDAGRGV TSYVIDTGVN INHKDFEKRA IWGKTIPLND EDLDGNGHGT HCAGTIAS-- ---KHYGVAK NANVVAVKVL RSNGSGTMSD  394
ASPERG G---QQSTDY IYDTSAGEGT YAYVVDSGVN VDHEEFEGRA SKAYNAAGGQ -HVDSIGHGT HVSGTIAG-- ---KTYGIAK KASILSVKVF QGESSST-SV  229
PROK   SSTSPGTSTY YYDESAGQGS CVYVIDTGIE ASHPEFEGRA QMVKTYYYS- -SRDGNGHGT HCAGTVGS-- --GRTYGVAK KTQLFGVKVL DDNGSGQYST  107
SUBTI  GIPLIKADKV QAQGFKGANV KVAVLDTGIQ ASHPDLNVVG G-ASFVAGEA YNTDGNGHGT HVAGTVAALD NTTGVLGVAP SVSLYAVKVL NSSGSGSYSG  105

Isp6   VIAGVEWTVR -HHKSS---- -G-KKTSVG- NMSLGGG-NS FVLDMAVDSA VTNGVIYAVA AGNEYDDACY SS---PAASK KAITVGASTI NDQMAYFSNY  378
CEREVI VVKGVEYAAK AHQKEAQEKK KG-FKGSTA- NMSLGGG-KS PALDLAVNAA VEVGIHFAVA AGNENQDACN TS---PASAD KAITVGASTL SDDRAYFSNW  488
ASPERG ILDGFNWAAN -DIVSK---- -K-RTSKAAI NMSLGGG-YS KAFNDAVENA FEQGVLSVVA AGNENSDAGQ TS---PASAP DAITVAAIQK SNNRASFSNF  318
PROK   IIAGMDFVAS -DKNNR---- -NCPKGVVA- SLSLGGG-YS SSVNSAAARL QSSGVMVAVA AGNNNADARN YS---PASEP SVCTVGASDR YDRRSSFSNY  196
SUBTI  IVSGIEW--- ----AT---- -T-NGMDVI- NMSLGGASGS TAMKQAVDNA YARGVVVVAA AGNSGNSGST NTIGYPAKYD SVIAVGAVDS NSNRASFSSV  191
                                  *
Isp6   GSCVDIFAPG LNILSTWIGS NTSTNTISGT SMATPHVAGL SAYYLGLHPA ASASEVKDAI IKMGIHDVLL SIPVGSSTIN LLAFNGAQE- ----------  467
CEREVI GKCVDVFAPG LNILSTYIGS DDATATLSGT SMASPHVAGL LTYFLSLQPG SDSEFFELGQ DSLTPQQLKK KLIHYSTKDI LFDIPEDTPN VLIYNGGGQD  588
ASPERG GKVVDVFAPG QDILSAWIGS SSATNTISGT SMATPHIVGL SLYLAALENL DGPAAVTKRI KELATKDVVK DV---KGSPN LLAYNGNA-- ----------  403
PROK   GSVLDIFGPG TD-LS-WIGG --STRSISGT SMATPHVAGL AAYLMTLGKT TAASACR-YI ADTANKGDLS NIPFGTFNLA YNNYQA---- ----------  277
SUBTI  GAELEVMAPG AGVYSTY--P TNTYATLNGT SMASPHVAGA AALILSKHPN LSASQVRNRL SSTATYLGSS FYYGKGLINV EAAAQ----- ----------  274

Isp6   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- 467
CEREVI LSAFWNDTKK SHSSGFKQEL NMDEFIGSKT DLIFDQVRDI LDKLNII--- ---------- ---------- ---------- ----- 635
ASPERG ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- 403
PROK   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- 277
SUBTI  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- 274
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Fig. 5. Comparison of amino-acid sequences of the *isp6* gene product and proteinases in other species. The entire sequences of the gene products of *isp6*, proteinase B in *S. cerevisiae* (CEREVI) (Moehle et al. 1987), alkaline proteinase in *A. oryzae* (ASPRERG) (Tatsumi et al. 1989), proteinase K in *T.album* (PROK) (Jany et al. 1986) and Carlsberg subtilisin in *B. subtilis* (SUBTI) (Smith et al. 1968) are aligned. The positions showing amino-acid identity are indicated by *shadowing*. Amino-acid residues on the catalytic active site are marked with *asterisks*. The N-termini of proteinase B in *S. cerevisiae* and alkaline proteinase in *A. oryzae* are processed between the two residues indicated by *underlining*. The C-terminus of proteinase B is also processed between the two residues indicated by *double underlining*

Based on microscopic observation, arrest in the *isp3* mutant seemed to take place at various stages after conjugation (Fig. 7). When the cells were stained with DAPI, two to four nuclei per cell were recognized (data not shown), which means that the first meiotic division had been successfully accomplished.

In the *isp6* disruptant, on the other hand, conjugation of the haploid cells of opposite mating types did not occur (Fig. 7). This coincides with the fact that transcription of *isp6* drastically increases 4 h after the induction of sexual differentiation, when most of the cells are conducting premeiotic cell division.

Discussion

In the present study, *isp3, 4, 5, 6, and 7*, corresponding to cDNA clones isolated by subtractive screening, were identified as genes that are preferentially transcribed during the sexual differentiation process. This was evidenced by the fact that transcripts of *isp* genes began to accumulate after induction at various stages of differentiation in a gene-specific manner, whereas no or little transcripts were observed under premeiotic conditions. Furthermore, transcription profiles of *isp* genes in JY274 and JY275 seem to reflect part of a complex cascade of regulation, since different profiles in terms of time specificity and quantity were observed between these two strains. The analysis of expression mechanisms of *isp* genes would contribute toward understanding the whole system of regulation that controls the sexual differentiation process.

The amino-acid sequence of the putative gene product of *isp6* showed significant homology to that of a serine proteinase, termed proteinase B, in *S. cerevisiae*. It was reported that proteinase B in *S. cerevisiae* is translated as a proenzyme consisting of 635 amino-acid residues, followed by processing of both N- and C-termini, producing an active mature polypeptide of 293 amino-acids (Fig. 5;

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A  Isp3
                10         20         30
  1 MGLGNLCSYK QDDSLDILQK KVLIDAFNKV
 31 TIDGKPNVQH QQPTYWYPPP PPRHHKEHKK
 61 SHHHWEYSSD DEESCEKKKP KCCEKKKPKC
 91 CESEQNNGCG RRNQLARRLA FLGSFGDGDC
121 DGCNEAFTVT GPITYFRTCP DPLTGITPAV
151 AAAAATPAAA ATPATPAAAA TPAAPAA

B  Isp4
                10         20         30
  1 MIGSINESPI EEHMNDSPST KEKADSVDIS
 31 DYIVSHSDDS LSKDIKKDTK SFLDVEHGEI
 61 STVDEFEEDS PYPEVRAAVP PTDDPSMPCN
 91 TIRMWTIGLI YSTVGAAVNM FFSLRNPTVT
121 LSVLISELLA YPALQIWDLI FPDREFRIGR
151 LKFNFKPGPF NVKEHALIVV MSSVSFGNAY
181 STDIILAQRV HYKQRFGFGY EICLTLATQL
211 IGYGLAGLSR RLLVRPASML WPVNLVQCTL
241 IKTLHRKDLR NAVANGWRIS PFRFFLYVFI
271 ASFIWNWFPS YIFQALSLFA WVTWIRPNSP
301 TVNQIFGEST GISILPMTFD WNQISAYILS
331 PLMAPADALM NILLGVILFF WIVTPALNFT
361 NTWYGDYLPI SSSGIIDHFG NSYNVTRILT
391 KDATFDLDAY QNYSPIFMST TYALAFGLSF
421 ASITSVIFHV ILYHGKEIYD RLRDPPAPDI
451 HEKLMKAYDE VPFYWYLSVF LAFFGMMMGT
481 IYGWKTETPW WVIIVGVIFS AVWFIPIGIV
511 QAITNIQLGL NVFTEFIVGY MYPGRPLAMM
541 IFKTVGYITM TQGLAFAADL KFGHYMKLPP
571 RIMFYTQMIA TIWSCFVQIG VLDWALGNID
601 NVCQADQPDN YTCPNATVFF NSSVIWGVIG
631 PKRMFSGKNT YTGLQYFWLA GVLGTILFWA
661 LWKKWPQKWW GQLNGPLIFG GTGYIPPATP
691 VNYLAWSGIG LFFNYYLKKI FADWWQKYNF
721 TLSALDTGTQ LSVIILFFCL QLPMVNFPDW
751 WGNDGAFNTL DATGAAVRKL VNESAR

C  Isp7
                10         20         30
  1 MLSQLIERST QYVREASLEE QNRIMPLIDF
 31 GPYVNQEPGA HERIIQQLRA ACESTGFFQI
 61 VNSPISPDVV KNAFRASKQF FELPFDEKLT
 91 LSKDMFSNRG YELMEDFVLE GEEDSSSPLE
121 ISGIDFEAGS YPGEAPLPPS SIGYVLPPSS
151 LANGEGSSMF DADMTTSNAV AHGDESISNE
181 FRESFYFGND NLSKDRLLRP FQGPNKWPST
211 AGSSFRKALV KYHDQMLAFA NHVMSLLAES
241 LELSPDAFDE FCSDPTTSIR LLRYPSSPNR
271 LGVQEHTDAD ALTLMSQDNV KGLEILDPVS
301 NCFLSVSPAP GALIANLGDI MAILTNNRYK
331 SSMHRVCNNS GSDRYTIPFF LQGNIDYVVA
361 PLPGLGPSTA EPIAVEDLLR DHFQNSYTSH
391 TTSLEVA
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Fig. 6 A–C. Amino-acid sequences of the putative products of *isp3*, *4*, and *7*. Translated amino-acid sequences of the ORFs in pMI3, 4, and 7 are shown as the putative products of: **A**, *isp3* ; **B**, *isp4* ; and **C**, *isp7*. The characteristic feature in the sequence of Isp3 is indicated by *underlining* (see text)

and Moehle et al. 1987). The N-terminus of alkaline proteinase in *A. oryzae* is also known to undergo processing (Fig. 5; Tatsumi et al. 1989). Taking into consideration the fact that homology among the three enzymes extends into the N-terminal prosequence regions, it is very probable that the N-terminal portion of the *isp6* gene product is processed after translation.

Disruption of *isp6* caused complete loss of the ability to produce spores. Most of the cells seemed to arrest before the occurrence of conjugation. In *S. cerevisiae*, proteinase B takes part in the degradation of somatic proteins to produce amino-acids for the synthesis of sporulation-

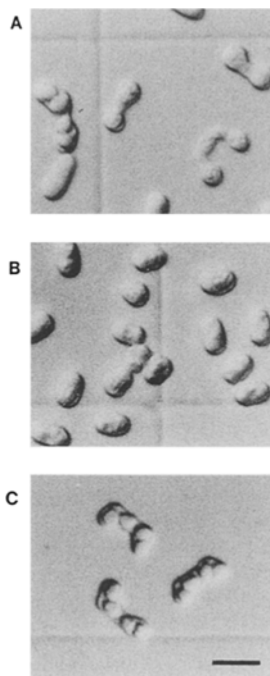

Fig. 7 A–C. Effects of disruption of *isp3* and *isp6*. SS003 (**A**), SS006 (**B**), and JY800 (**C**) were grown in YPD medium and sexual differentiation was induced by transferring the cells into SPA as described in the Materials and methods. After 24 h, cells were observed under a Nomarski differential-interference-contrast microscope. Bar=10 μm

Table 2. Spore analysis of gene disruption mutants of *isp*. Cells were grown in YPD medium, and transferred to SPA agar plates to induce sexual differentiation. After 24 h, the efficiency of spore formation was tested by random spore analysis. In each experiment, more than 250 cells were examined, n.d., not determined

Strain	Genotype	Sporulation (%)	Spore viability (%)
JY800	WT	70.5	87.5
SS003	*isp3::ura4*	45.5	81.3
SS004	*isp4::ura4*	68.6	87.5
SS005	*isp5::ura4*	72.7	84.3
SS006	*isp6::ura4*	0.0	n.d.
SS007	*isp7::ura4*	71.7	81.3

specific proteins, and it is indispensable for sporogenesis (Klar and Halvorson 1975; Betz and Weiser 1976). In *S. pombe*, the activity of serine proteinase increases after the cells are transferred into sporulation medium, while no activity is detected in the *isp6* disruption mutant (S. Uritani, personal communication).

Therefore, it is strongly suggested that the product of *isp6* is a serine proteinase concerned with protein reconstruction during the phase transition between vegetative growth and sexual differentiation.

The present primary structural analysis of pMI5 indicates that *isp5* codes for an amino-acid permease. Production of Gap1, a general amino-acid permease in *S. cerevisiae*, is induced by nitrogen starvation (Jauniaux and Grenson 1990), as is observed with *isp5*. *GAP1* and *isp5*, both of which share significant sequence similarity, might thus participate in urgent amino-acid transport in response to deprivation of a nitrogen source.

In contrast with *isp5* and *isp6*, we could not find any sequences in the databases which show obvious similarity

to the nucleotide and the translated amino-acid sequences of *isp3*, *4*, and *7*. In the *isp3* disruption mutant, significant loss of spore-forming ability was observed, while no effect was seen when deletions were introduced into the coding regions of *isp4* and *isp7*. The putative product of *isp3* shares a structural feature, short stretches of alanine residues interrupted by threonine and proline, with a translated amino-acid sequence of the cDNA clone, I3, whose expression is specific to developing microsporocytes in *B. napus* (Roberts et al. 1991). Considering the fact that transcription of both *isp3* and the I3 gene occurs at a similar period of development, together with the abundance of *isp3* transcripts found in the cells, we speculate that the gene product of *isp3* may be a structural protein which is necessary for spore formation.

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