Molecular Biology of the Cell
Vol. 6, 1185–1195, September 1995

Schizosaccharomyces pombe zfs1^{+} Encoding a Zinc-Finger Protein Functions in the Mating Pheromone Recognition Pathway

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Submitted April 3, 1995; Accepted July 14, 1995
Monitoring Editor: Michael H. Wigler

We isolated the *Schizosaccharomyces pombe zfs1* gene as a multicopy suppressor of the sterility caused by overexpression of a double-stranded RNase. The deduced *zfs1* gene product of 404 amino acids showed similarity to a mouse growth factor-inducible nuclear protein Nup475. Its C-terminal region carried two putative zinc-fingers, both of which should be intact for the protein to be functional as the suppressor. This protein appeared to localize in nuclei. Disruption of *zfs1* was not lethal but conferred deficiency in mating and sporulation. Activation of transcription in response to the mating pheromone signaling was greatly reduced in the *zfs1*-disrupted cells. The mating deficiency of the *zfs1*-disruptant was suppressed partially by overexpression of either *gpa1*, *ras1*, *byr1*, or *byr2*, which are involved in the transmission of the pheromone signal. Disruption of *zfs1* reduced both hypersensitivity of the *ras1*^{Val17} mutant to the mating pheromone and uncontrolled mating response caused by mutational activation of Gpa1, the G protein α subunit coupled to the mating pheromone receptors. However, overexpression of *zfs1* could not bypass complete loss of function of either *gpa1*, *ras1*, *byr1*, or *byr2*. These observations indicate that the function of *zfs1* is involved in the mating pheromone signaling pathway, and are consistent with its function being required to fully activate a factor in this pathway, either directly or indirectly.

INTRODUCTION

Schizosaccharomyces pombe cells proliferate by cell division when nutrients are plentiful in the environment. If they are starved, particularly for a nitrogen source (Egel, 1971), cells of the two opposite mating types mate and the resultant zygotes follow the sporulation pathway (Egel, 1973). The single Ras homologue in *S. pombe*, Ras1, is required for the response to mating pheromones (Fukui *et al.*, 1986a; Nadin-Davis *et al.*, 1986). Cells defective in the *ras1* gene secrete mating pheromones but cannot respond to them (Fukui *et al.*, 1986b; Leupold *et al.*, 1991), whereas cells carrying an activated allele of *ras1* are hypersensitive to the pheromones and form abnormally long conjugation tubes (Fukui *et al.*, 1986a,b; Nadin-Davis *et al.*, 1986). In cells defective in *ras1*, expression of *mat1-Pi*, which is known to be induced by acceptance of the mating pheromone (Kelly *et al.*, 1988; Nadin-Davis and Nasim, 1990), is greatly reduced (Nielsen *et al.*, 1992). Based on these phenotypes it has been proposed that the Ras pathway modulates sensitivity to the mating pheromone signal in *S. pombe* (Hughes and Yamamoto, 1993).

Three protein kinases, namely Byr2 (Wang *et al.*, 1991), Byr1 (Nadin-Davis and Nasim, 1988, 1990), and Spk1 (Toda *et al.*, 1991; Gotoh *et al.*, 1993), have been shown to function downstream of *ras1*. These kinases are homologous to a set of kinases that constitute the MAP kinase cascade in higher eukaryotes (Neiman *et al.*, 1993; Nishida and Gotoh, 1993). The *gpa1* gene of *S. pombe* encodes a G protein α subunit and loss of its function blocks both mating and sporulation (Obara *et al.*, 1991). Introduction of an activated form of Gpa1

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into heterothallic haploid cells induces morphological changes suggestive of a mating response in the absence of the mating partner (Obara *et al.*, 1991). Genetic evidence placing Gpa1 upstream of the MAP kinase cascade has been described (Neiman *et al.*, 1993; Xu *et al.*, 1994). These observations strongly suggest that the Gα subunit encoded by *gpa1* is coupled with the mating pheromone receptors and transduces the pheromone signal to the downstream MAP kinase cascade. Thus, both Ras1 and Gpa1 appear to function upstream of the MAP kinase cascade in *S. pombe* cells, as previously noted (Xu *et al.*, 1994). In particular, direct physical interaction has been evidenced between Ras1 and Byr2 (Masuda *et al.*, 1995).

Meanwhile, we previously characterized the *S. pombe pac1* gene, which encodes an RNase III-like dsRNase (Iino *et al.*, 1991). This gene was originally isolated as a multicopy suppressor of the *pat1*^{ts} mutation, which induces ectopic meiosis at the restrictive temperature (Beach *et al.*, 1985; Iino and Yamamoto, 1985a,b; Nurse, 1985), and was subsequently shown to be able to block physiological mating and meiosis if overexpressed in the wild-type cells (Iino *et al.*, 1991). To isolate possible positive regulators of mating and meiosis, we set out to screen for multicopy suppressors of the sterility of a strain overexpressing *pac1*. In this report we characterize one of the *S. pombe* genes thus isolated. Despite its isolation strategy, this gene, named *zfs1*, did not appear to function in close association with *pac1*. However, genetic analysis indicated that the function of *zfs1* is involved in the mating pheromone recognition pathway.

MATERIALS AND METHODS

Strains and Media

S. pombe strains used in this study are listed in Table 1. General genetic procedures for *S. pombe* were performed according to the method of Gutz *et al.* (1974). Yeast media YPD and SD (Sherman *et al.*, 1986), SSA (Egel and Egel-Mitani, 1974), SPA and MEA (Gutz *et al.*, 1974), and PM and its nitrogen-free version PM-N (Beach *et al.*, 1985; Watanabe *et al.*, 1988) were described previously. Transformation (Beach *et al.*, 1982; Okazaki *et al.*, 1990) and gene disruption (Rothstein, 1983; Fukui *et al.*, 1986a) were done as described.

Recombinant DNA Techniques

Recombinant clones were prepared and handled according to the method of Sambrook *et al.* (1989), unless otherwise noted.

Expression Vectors

To overexpress genes in *S. pombe* cells, four kinds of expression vectors were used. pEVP11 (Russell, 1989) and pART1 (McLeod, 1987) carry the *adh1* promoter, whereas pREP1 and pREP41 carry the *nmt1* promoter (Maundrell, 1993). The *nmt1* promoter is repressed in the presence of thiamine, but is stronger than the *adh1* promoter if derepressed. pREP41 has an altered *nmt1* promoter, the derepressed activity of which is only 15% of that of the intact *nmt1* promoter but is comparable to that of the *adh1* promoter (Basi *et al.*, 1993). Because overexpression of *zfs1* from pREP1 appeared to be

Table 1. *S. pombe* strains used in this study

Strain	Genotype
JY333	h^- *ade6-M216 leu1*
JY362	h^+/h^- *ade6-M210/ade6-M216 leu1/leu1*
JY450	h^{90} *ade6-M216 leu1*
JY765	h^+/h^- *ade6-M210/ade6-M216 leu1/leu1 ura4-D18/ura4-D18*
JY900	h^{90} *ade6-M210 leu1 ras1*^{val17}*<<LEU2*
JZ46	h^{90} *ade6-M210 ura4-D18*
JZ467	h^{90} *ade6-M210 leu1::(pac1-o.e. + ura4*$^+$*) ura4-D18*
JZ522	h^{90} *ade6-M216 leu1 ura4-D18 ras1::ura4*$^+$
JZ971	h^{90} *ade6-M216 leu1 ura4-D18 zfs1::ura4*$^+$
JZ974	h^- *ade6-M216 leu1 ura4-D18 zfs1::ura4*$^+$
JZ976	h^+/h^- *ade6-M210/ade6-M216 leu1/leu1 ura4-D18/ura4-D18 zfs1::ura4*$^+$*/zfs1::ura4*$^+$
JX5	h^{90} *ade6-M210 leu1 ura4-D18 byr3::ura4*$^+$
JX14	h^{90} *ade6-M216 leu1 ura4-D18 ras1*^{val17}*<<LEU2 zfs1::ura4*$^+$
JX191	h^{90} *ade6-M216 leu1 ura4-D18 gpa1::ura4*$^+$

slightly toxic to the host cells, pREP41 was employed in most physiological experiments.

Construction of a pac1-overexpressing Strain

A 1.7-kb *Bcl*I–*Hin*dIII fragment that carried the entire *pac1* open reading frame (ORF) but lacked its authentic promoter was connected to the *S. pombe adh1* promoter on an expression vector pEVP11 (Russell, 1989). An *Sph*I site in the upstream of the *adh1* promoter was altered to a *Hin*dIII site, and the *adh1-pac1* composite gene was cut out with *Hin*dIII restriction endonuclease. The composite gene was then inserted at the *Hin*dIII site of pLU′, which is a plasmid designed to integrate the insert into the *leu1* locus of the *S. pombe* genome (Mochizuki and Yamamoto, 1992). A *Bam*HI–*Xho*I fragment carrying the composite gene and a *ura4*$^+$ cassette adjacent to it, the combination of which was flanked by segments of the *leu1* gene, was excised from the resultant plasmid. JZ46 (h^{90} *ade6-M210 ura4-D18*) was transformed with this fragment and Ura$^+$ Leu$^-$ transformants were selected. Successful integration of the composite gene at the *leu1* locus was confirmed by Southern blotting in one of the transformants, which was designated JZ467.

Nucleotide Sequence Determination

The nucleotide sequence of a 3.4-kb *Eco*RV–*Pst*I fragment carrying the *zfs1* gene was determined by the dideoxy chain-termination method (Sanger *et al.*, 1977). Subclones for sequencing were prepared by progressive deletion of the fragment with exonuclease III and S1 nuclease (Takara Shuzo, Kyoto, Japan), according to Henikoff (1984). All parts of the sequence shown in Figure 2 were determined in both directions at least once.

In Vitro Mutagenesis

In vitro site-directed mutagenesis of the *zfs1* gene was performed according to the method of Kunkel (1985) using designed synthetic oligonucleotides.

Gene Disruption

Disruption of the *zfs1* gene was done essentially as described originally (Rothstein, 1983). A *Bam*HI cutting site was introduced in the immediate upstream of the initiation codon of the cloned *zfs1* gene, by using a synthetic oligonucleotide 5′-AGAATAAACCATG-

GATCCAAGAATAAA-3′, in which the *Bam*HI recognition sequence is shown in bold and the initiation codon (antisense) is underlined. Then, a *Bam*HI–*Eco*RV fragment, which covered the entire *zfs1* ORF but little more than that, was replaced by a *ura4*+ cassette (Grimm *et al.*, 1988). A diploid strain JY765 was transformed by an *Eco*RV–*Pst*I fragment that carried this disruption construct and Ura+ transformants were selected. Precise replacement of the genomic *zfs1*+ gene by the disrupted allele was confirmed by Southern blotting.

Preparation of Antibodies

A GST-Zfs1 fusion protein was expressed in *Escherichia coli* using pGEX-KG (Guan and Dixon, 1991), as follows. A 0.6-kb *Hinc*II–*Eco*RV fragment that covered the C-terminal half of Zfs1 was cloned into pGEX-KG in frame. Expression of the fusion protein was induced in *E. coli* BL21 (DE3) by the addition of 4 0.1 mM isopropyl-1-thio-β-D-galactoside. After incubation for 4 h, a cell extract was prepared and the fusion protein was purified by using a GSH-agarose column. The protein preparation (1 mg) was injected into a rabbit with Freund's adjuvant. The antigen was re-injected at 1-mo intervals, and sera were sampled 2 wk after each injection to check the titer. Antibodies were affinity purified by incubating with a polyvinylidene difluoride membrane blotted with the antigen.

Western Blotting and Isolation of Nuclei

For Western blot analysis, proteins separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred electrophoretically to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA), and processed according to a standard protocol (Towbin *et al.*, 1979). Nuclei were isolated from *S. pombe* cells by Percoll gradient centrifugation as described previously (Hirano *et al.*, 1988).

Preparation of RNA from S. pombe Cells and Northern Blot Analysis

S. pombe cells under various experimental conditions were harvested by centrifugation and broken by vortexing vigorously with glass beads in a buffer solution containing 0.2 M Tris-HCl (pH 7.5), 0.5 M NaCl, 0.01 M EDTA, and 1% SDS. After repeated extraction with phenol-chloroform (1:1), RNA was recovered by ethanol precipitation. For Northern blot analysis, 10 μg of each RNA preparation was denatured with formamide, separated by formaldehyde gel electrophoresis (Sambrook *et al.*, 1989), and blotted to a membrane (GeneScreen plus, Dupont, Wilmington, DE). The following probes were used to detect transcription of respective genes: *ste11*, a 1.8-kb *Hin*dIII–*Mlu*I fragment carrying the entire ORF (Sugimoto *et al.*, 1991); *mat1-Pc* and *mat1-Pi*, a 0.9-kb *Hin*PI–*Mlu*I fragment that covers the two ORFs nearly completely (Kelly *et al.*, 1988); *mam2*, a 3.5-kb *Hin*dIII fragment carrying the entire ORF (Kitamura and Shimoda, 1991); and *zfs1*, a 1.2-kb *Bam*HI–*Eco*RV fragment that covers the entire ORF (this study). The *Bam*HI end of the *zfs1* probe was an artificial restriction site produced to aid disruption of the gene. Probe DNA was labeled with [α-^{32}P]dCTP by the random priming method.

RESULTS

Isolation and Sequence Analysis of the zfs1 Gene

The *S. pombe pac1* gene encodes a double-stranded RNase and its overexpression from a high copy plasmid inhibits sexual development (Iino *et al.*, 1991). JZ467, a *pac1*-overexpressing strain constructed as described in MATERIALS AND METHODS, was inert in mating and sporulation. Northern blot analysis confirmed that the level of *pac1* mRNA was elevated in JZ467, and Western blot analysis indicated the *pac1* gene product was indeed overproduced in it (our unpublished data).

We then screened for multicopy suppressors of the mating and sporulation deficiency of JZ467 using an *S. pombe* genomic library based on the multicopy vector pDB248′ (Beach *et al.*, 1982). A suppressor clone pMS-25 isolated in this screen carried a novel *S. pombe* gene, which we named *zfs1*. JZ467 cells transformed with pMS-25 could mate and sporulate on SSA. The mating frequency of the transformant was 39%, whereas that of JY467 cells carrying the vector was virtually zero. The *S. pombe* genomic DNA inserted in pMS-25 was 7.0-kb in length. The region responsible for the suppression was delimited to a 2.6-kb *Eco*RV fragment by subcloning (Figure 1). Nucleotide sequence analysis revealed a 1.2-kb-long ORF on this fragment (Figure 2). The predicted *zfs1* gene product consisted of 404 amino acid residues. No intron consensus was found in this region. Genomic Southern hybridization using the *zfs1* ORF as a probe suggested that the gene is unique in the *S. pombe* genome (our unpublished data).

The deduced *zfs1* gene product was compared with the protein sequences in the NBRF data base, and was found to show the highest similarity to a mouse growth factor-inducible nuclear protein Nup475 (DuBois *et al.*, 1990). The C-terminal 127 amino acids of the *zfs1* product shared 37.0% identity with Nup475 (Figure 3), and this region carried two putative zinc-finger motifs $CX_8CX_5CX_3H$.

Cells Defective in zfs1 Have a Reduced Mating Ability

One *zfs1* allele of a diploid strain JY765 was disrupted as described in MATERIALS AND METHODS.

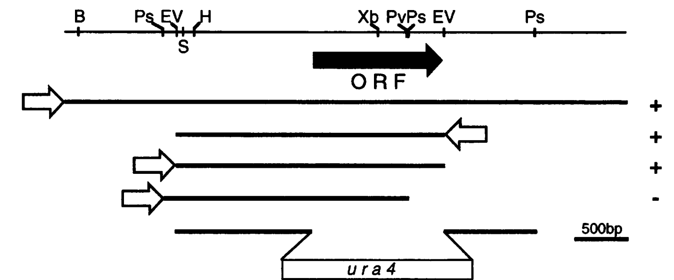

Figure 1. A restriction map of the *zfs1* locus. The insert DNA in plasmid pMS-25 is shown on the top with the solid arrow indicating the extent and direction of the *zfs1* ORF. The ability of various subclones to suppress the sterility of the *pac1*-overproducer is indicated as (+) and (−). Open arrows indicate the direction of transcription from a cryptic promoter on the vector. Restriction sites are abbreviated as follows: B, *Bam*HI; EV, *Eco*RV; H, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*II; S, *Sac*I; and Xb, *Xba*I.

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-516 TTTGCATTCAACTCGGATTTTTTCTTTGTTTGACGACTGTTTTTTGAAAAGGATTGCTTT -457
-456 GGGTCACAAACACAATACATCCATCTATTAAACTTTTGTCTTCTATACTTTTACTTCCTT -397
-396 CTCAAAATCTTTCCTTATATCATTGTGTGGTTTCTCTCCGTTTTCTTGTATCTTTTCTGT -337
-336 TCGACTTCTGAAAAAACAGCGTCTCTATACTATTGATTTTCATCTTAAATTTGCGTGTTT -277
-276 TCTTTTTAACTCTACGTTTCTCTCGTTTTTCTGCATATTACTTTCGGTGATTACCCTGTA -217
-216 ATTCTATTTTGAAGGTTGACGTTTTTTTCAAGTTCCTGATTCCATAATTCTATTTAACAC -157
-156 AAGTTTTTTACTGGAATTTTTTAAGTTATTCTGCGCAACCTTTTTTTGATTCATTTTACA -97
 -96 ACTTCTTTGTTCGTTTATAACTAATCTTCTATTGTATCACTTTTTTCTAGTGAACTTCAT -37
 -36 TAATTTATTGCGTGTCTGTTTTTATTCTTTTAACCATGGTTTATTCTCCTATGTCTCGA 24
   1                                     M  V  Y  S  P  M  S  R   8

  25 CCTCAAGTACCTCTGGCTTTTCGCCAATGGCCCCCCTACAATTATAAGAGTGATCCCCTT 84
   9 P  Q  V  P  L  A  F  R  Q  W  P  P  Y  N  Y  K  S  D  P  L   28

  85 GTCAACTCAAAACTATCTCAGTCTACTACGTCTGTTAATGCAGGACCCAGTTTAATAAGT 144
  29 V  N  S  K  L  S  Q  S  T  T  S  V  N  A  G  P  S  L  I  S   48

 145 CCATCATTCCTTGACTCCTACGCCAACTCCTCATCTTTACTTCACAAGCCATCTACAAAT 204
  49 P  S  F  L  D  S  Y  A  N  S  S  S  L  L  H  K  P  S  T  N   68

 205 TTGGGCAGTTTAAAGACATCTAGTCTTCTCGCCTCTGATGAAGTTTTCCCTTCTTCAGTC 264
  69 L  G  S  L  K  T  S  S  L  L  A  S  D  E  V  F  P  S  S  V   88

 265 ATGCCCCAGCTTCGTCCATTGGATTCAAGTTTATCTGTTTCTCCTGAAATGGACGGTTGG 324
  89 M  P  Q  L  R  P  L  D  S  S  L  S  V  S  P  E  M  D  G  W   108

 325 CCATGGCATCACAATTCTTCTTCTAACCCACAGGGATACGCATGGACACCTTCATTACTT 384
 109 P  W  H  H  N  S  S  S  N  P  Q  G  Y  A  W  T  P  S  L  L   128

 385 TCTTCCAACGCTACTAGCTATTTACATTCTGGTAGTTCTCCTCATGGAAACACAAGCAAT 444
 129 S  S  N  A  T  S  Y  L  H  S  G  S  S  P  H  G  N  T  S  N   148

 445 CATCCTTCACCTATATCATCCTTGGAGAGTCTTCCTTCTCGTAGTAGCACTGGTAGTGGT 504
 149 H  P  S  P  I  S  S  L  E  S  L  P  S  R  S  S  T  G  S  G   168

 505 AGTCTTGATTTTTCCGGCCTTGCCAATCTCCACGATGATTCCAAATCACTGGCCATGTCT 564
 169 S  L  D  F  S  G  L  A  N  L  H  D  D  S  K  S  L  A  M  S   188

 565 TTGAACATGGCTGGCGTCCCTGTATCCGTCGATGAGAACTCTCAAACTTCGTTTCCTTTT 624
 189 L  N  M  A  G  V  P  V  S  V  D  E  N  S  Q  T  S  F  P  F   208

 625 GTTCATGGTCAACCGGAATCTACCATGTCTAGAAAACCTAAGCTGTGCGTTCAATCGAAA 684
 209 V  H  G  Q  P  E  S  T  M  S  R  K  P  K  L  C  V  Q  S  K   228

 685 TCAATGACAAACATCCGCAACTCTGTTGCTAAACCGTCGTTAGTTAGACAATCGCATTCA 744
 229 S  M  T  N  I  R  N  S  V  A  K  P  S  L  V  R  Q  S  H  S   248

 745 GCGGGGGTTATTCCCATCAAGCCTACAGCATCGAATGCCTCAATTCGCAATGCTCCTTCC 804
 249 A  G  V  I  P  I  K  P  T  A  S  N  A  S  I  R  N  A  P  S   268

 805 AATTTATCAAAGCAATTTTCACCTTCGGGTAATAGTCCTCTTACTGAGGCTTCCAAACCT 864
 269 N  L  S  K  Q  F  S  P  S  G  N  S  P  L  T  E  A  S  K  P   288

 865 TTCGTTCCTCAACCATCAGCTGCAGGAGATTTTCGCCAAGCTAAGGGTTCAGCTAGCCAT 924
 289 F  V  P  Q  P  S  A  A  G  D  F  R  Q  A  K  G  S  A  S  H   308

 925 CCTCATGGTTCTGGCAGTAGTAATGGGGTAGCTCCCAACGGCAAGCGCGCATTATATAAG 984
 309 P  H  G  S  G  S  S  N  G  V  A  P  N  G  K  R  A  L  Y  K   328

 985 ACCGAGCCTTGCAAGAATTGGCAAATTAGTGGAACTTGCCGTTATGGCTCTAAATGTCAA 1044
 329 T  E  P  C  K  N  W  Q  I  S  G  T  C  R  Y  G  S  K  C  Q   348

1045 TTTGCGCATGGCAACCAGGAACTGAAAGAACCTCCTCGTCACCCAAAATACAAAAGCGAG 1104
 349 F  A  H  G  N  Q  E  L  K  E  P  P  R  H  P  K  Y  K  S  E   368

1105 CGTTGCCGATCTTTCATGATGTATGGTTATTGTCCTTACGGATTGCGATGCTGCTTTTTG 1164
 369 R  C  R  S  F  M  M  Y  G  Y  C  P  Y  G  L  R  C  C  F  L   388

1165 CATGATGAATCCAATGCTCAAAAAAGTGCAACTATTAAGCAATCTCCTTGATATCACATC 1224
 389 H  D  E  S  N  A  Q  K  S  A  T  I  K  Q  S  P  *            405

1225 AGCTTGTATAAGATGACTTTTGTTCTTTGTTGTTATGCAATCTGGGTGAGATTAATTTAT 1284
1285 TATGACGCTTACTTTCCTATCCTCCTTGGTTTTCTTATACGGTTATTATTTATATTGTTC 1344
1365 TTCTGTAACATTATGTGAGCTTTTCTTTTGCAATCGCCTTTACCTTGTTTTTATTTATGG 1404
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Figure 2. The nucleotide sequence of the *zfs1* gene and its deduced amino acid sequence. Numbering starts with the first methionine codon in the ORF for both DNA and amino acid sequences. The GenBank/EMBL/DDBJ accession number for the nucleotide sequence reported here is D49913.

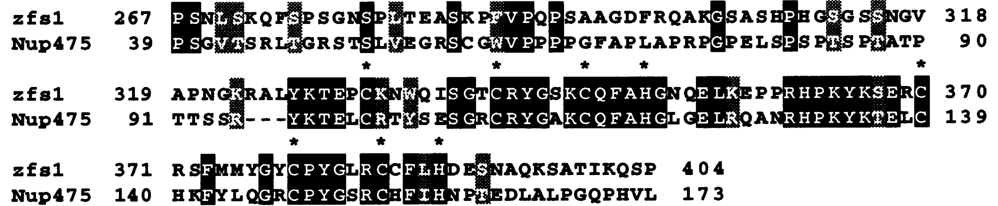

Figure 3. Comparison of the amino acid sequences of the deduced *zfs1* gene product and mouse Nup475. Amino acid residues identical between the two proteins are shown in white against black, whereas those showing conservative alteration are shown in white against gray. The cysteine and histidine residues highly conserved in zinc-finger motifs are marked with asterisks.

Proper integration of the disruption construct was confirmed by Southern blot analysis (our unpublished data). Asci generated by this diploid disruptant were dissected. Most of the asci gave four viable spores, two of which were Ura$^+$ and hence likely to be *zfs1*-disruptants. This suggested that the function of *zfs1* is not essential for vegetative cell growth. Spores carrying the *zfs1* null allele appeared to germinate normally. However, haploid *zfs1*-disruptants grew with a generation time of 3.5 h at 30°C in YPD medium, which was somewhat slower than the generation time of the wild-type cells under the same culture conditions (3 h). Growth retardation of *zfs1*-disruptants was more serious at a higher temperature (our unpublished observation).

We then examined whether the disruption of *zfs1* caused effects on sexual development. JZ971, a haploid homothallic strain carrying the *zfs1* null allele, was found to mate very poorly, and JZ976, a diploid strain homozygous for the *zfs1* null allele, was found to sporulate inefficiently (Table 2). We thus conclude that the function of *zfs1* is required for efficient mating and sporulation.

The Zinc-Fingers Are Essential for the Function of Zfs1

To determine whether the two zinc-finger motifs found in the C-terminal half of Zfs1 are necessary for the function of *zfs1 in vivo*, we constructed plasmids carrying mutant *zfs1* genes. The conserved histidine residue in each zinc-finger, which was supposed to be essential for the zinc-binding activity, was altered to isoleucine by in vitro site-directed mutagenesis (Kunkel, 1985). Although the parental plasmid pDB-zfs1 (WT) could suppress the sterile phenotype of JZ971 (*zfs1*Δ), three types of mutant plasmids that we constructed (carrying either the *zfs1-H351I* mutation, the *zfs1-H389I* mutation, or their combination) could not. Thus, both of the zinc-finger motifs should be intact for Zfs1 protein to fulfill its function.

Table 2. Effects of *zfs1* disruption on sexual development

Strain	Mating efficiency (%)	Sporulation efficiency (%)
JY450	75	—
JZ971 (*zfs1*$^-$)	6	—
JY362	—	72
JZ976 (*zfs1*$^-$)	—	26

Each strain was incubated on SSA plate for 2 days, and the mating or sporulation efficiency was examined.

Function of zfs1 Is Involved in the Pheromone Recognition Pathway

To further investigate the effect of *zfs1* disruption on sexual development, we examined expression of *S. pombe* genes essential for sexual development in a homothallic haploid *zfs1*-disruptant JZ971 (Figure 4). The *ste11* gene, which encodes a key transcription factor required for the expression of various genes that promote sexual development, has been shown to be activated transcriptionally by nitrogen starvation (Sugimoto *et al.*, 1991). Expression of *ste11* was in-

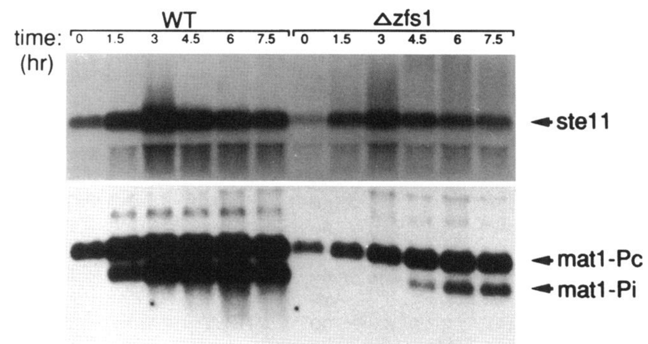

Figure 4. Northern blot analysis of three genes required for sexual development. JY450 (h^{90} wild type) and JZ971 (h^{90} *zfs1*-disruptant), grown to 5×10^6 cells/ml in PM medium, were shifted to nitrogen-free PM-N medium and cells were harvested at intervals as indicated. RNA was prepared from each aliquot and analyzed as described in MATERIALS AND METHODS.

duced in the *zfs1*-disruptant by nitrogen starvation nearly as strongly as in the wild type (Figure 4). Expression of *mat1-Pc*, which is another gene transcriptionally activated under nitrogen starvation (Kelly *et al.*, 1988), was similarly induced. In contrast, expression of *mat1-Pi*, which calls for the mating pheromone signaling in addition to nitrogen starvation (Kelly *et al.*, 1988; Nadin-Davis and Nasim, 1990), was considerably reduced and delayed in the *zfs1*-disruptant (Figure 4). Expression of *mam2*, which encodes the receptor for P-factor (Kitamura and Shimoda, 1991), is also known to be greatly enhanced by the mating pheromone signaling (Xu *et al.*, 1994). Transcription of *mam2* was suppressed in the *zfs1*-disruptant, even though the strain was homothallic (Table 3). These results suggested that the function of Zfs1 is required to optimize the transmission of the mating pheromone signaling. This possibility was further supported by the following analyses.

Functional Relation between Zfs1 and Gpa1

We examined possible epistasis between *zfs1* and *gpa1*, the latter of which encodes Gα coupled to the mating pheromone receptors (Obara *et al.*, 1991). The homothallic *zfs1*-disruptant JZ971 was transformed with pART1-gpa1, which carries the wild-type *gpa1* allele. The transformed cells could mate rather efficiently (Table 4), indicating that *gpa1* can be a high copy suppressor of *zfs1*Δ. However, overexpression of *zfs1* did not suppress the sterility of *gpa1*Δ cells at all. Consistently, no increase of *mam2* transcription was observed in *gpa1*Δ cells transformed with a *zfs1*-overexpressing plasmid pREP41-zfs1 (Table 3).

As previously demonstrated (Obara *et al.*, 1991), a heterothallic strain JY333 (h^- wild type) transformed with pART1-gpa1QL, which carries an activated *gpa1* allele (*gpa1-Q244L*), extended conjugation tubes under nitrogen starvation in the absence of acceptance of the mating pheromone (Figure 5B), whereas the same strain transformed with pART1-gpa1 did not exhibit such a sexual response (Figure 5A). In contrast, JZ974 (h^- *zfs1*Δ) did not extend conjugation tubes visibly under nitrogen starvation even if it was transformed with pART1-gpa1QL (Figure 5D). These results support the view that Zfs1 is required for the proper transmission of the pheromone signal.

Table 3. Expression of the *mam2* gene in various strains overexpressing *zfs1*

Strain	zfs1 overexpression	Relative *mam2* expression
wild type	no	1.00
zfs1$^-$	no	0.28
zfs1$^-$	yes	0.86
gpa1$^-$	no	0.17
gpa1$^-$	yes	0.09
ras1$^-$	no	0.43
ras1$^-$	yes	0.52

The host strains are JY450 (WT), JZ971 (*zfs1*$^-$), JX191 (*gpa1*$^-$), and JZ522 (*ras1*$^-$). Each strain was transformed with either the vector pREP41 or pREP41-zfs1. RNA was extracted from cells starved for nitrogen for 6 h. After hybridization, expression of *mam2* in each strain was quantitated using a Bioimage Analyzer (Fujix BAS2000).

Table 4. Mating efficiency of the *zfs1*-disruptant transformed with various multicopy plasmids

Gene on the multicopy plasmid	Mating efficiency (%)
None	4
zfs1	66
gpa1	55
ras1	19
byr1	17
byr2	43

The *zfs1*$^-$ disruptant (JZ971) was transformed with multicopy plasmids carrying the genes indicated. The vector used for *zfs1* and *ras1* was pDB248′ and that used for the others was pART1. The *zfs1* and *ras1* genes on the plasmid were expressed from their own promoters. Transformants were incubated on SSA plate for 2 days and their mating efficiency was examined.

Functional Relation between Zfs1 and Ras1

The *zfs1*-disruptant showed little mating response under nitrogen-starved conditions (Figure 6B), where wild-type *S. pombe* cells could mate and form zygotes (Figure 6A). In contrast, cells carrying the $ras1^{val17}$ mutation, which fixes Ras1 protein in the activated state, are known to be hyper-responsive to the mating pheromone (Fukui *et al.*, 1986b; Nadin-Davis *et al.*, 1986). They extend conjugation tubes excessively if exposed to the mating pheromone and eventually mate very poorly (Figure 6C). To investigate possible epistasis between *zfs1* and *ras1*, we constructed a double mutant carrying *zfs1*Δ and $ras1^{val17}$. The double mutant did not show the excessive mating response (Figure 6D). However, if $ras1^{val17}$ is overexpressed in *zfs1*Δ, the phenotype was closer to $ras1^{val17}$ (our unpublished data). Even overexpression of the wild-type *ras1* allele was able to suppress the mating defect of *zfs1*Δ partially (Table 4).

Overexpression of *zfs1* suppressed the reduced mating ability of a strain overexpressing *gap1*, which encodes the Ras1 GTPase-activating protein and downregulates the Ras1 activity (Imai *et al.*, 1991). However, overexpression of *zfs1* could not suppress the mating deficiency of the *ras1*Δ mutant, which lacks the Ras1

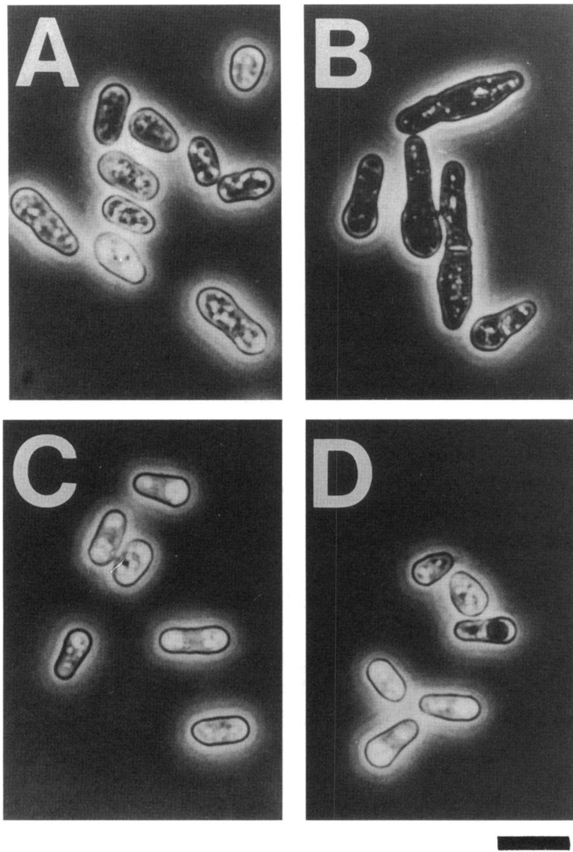

Figure 5. Cell morphology of various transformants placed under nitrogen starvation. (A) JY333 with pART1-gpa1; (B) JY333 with pART1-gpa1QL; (C) JZ974 with pART1-gpa1; and (D) JZ974 with pART1-gpa1QL. Cells were incubated on SPA medium for 2 days. Phase-contrast micrographs are shown. Bar, 10 μm.

activity completely. Again consistently, no significant increase of *mam2* transcription was observed in *ras1*Δ cells transformed with a *zfs1*-overexpressing plasmid pREP41-zfs1 (Table 3).

Functional Relation of Zfs1 to byr1, byr2, and byr3

We further examined epistasis between *zfs1* and either *byr1*, *byr2*, or *byr3*. It has been shown that overexpression of each *byr* gene can suppress sporulation deficiency of the diploid *ras1*Δ mutant but cannot suppress mating deficiency of the haploid *ras1*Δ mutant (Nadin-Davis and Nasim, 1988; Wang *et al.*, 1991; Xu *et al.*, 1992). The *byr1* and *byr2* genes, respectively, encode a MAP kinase kinase homologue and a MAP kinase kinase kinase homologue (Neiman *et al.*, 1993; Nishida and Gotoh, 1993), which are thought to function as signal transmitters downstream of both Ras1 and Gpa1 (Xu *et al.*, 1994). We overexpressed either *byr1* or *byr2* in the *zfs1*-disruptant. The results indicated that overexpression of each gene can suppress the mating defect of the disruptant (Table 4). However, no suppression of sterility was observed when *zfs1* was overexpressed in either a *byr1*Δ or a *byr2*Δ strain, as judged from the mating efficiency.

The *byr3* gene encodes a protein with seven zinc-finger domains ($CX_2CX_4HX_4C$), the functions of which are implicated in the mating pheromone signaling pathway, although not precisely specified (Xu *et al.*, 1992). The phenotype of the *zfs1*-disruptant is reminiscent of that of the *byr3*-disruptant in some aspects: disruption of *byr3* causes a decrease in mating efficiency but it is much less deleterious for sporulation. The mating defect of the *byr3*-disruptant can be partially restored by overexpressing *ras1* or *byr2*. Furthermore, a fusion protein carrying the Byr3 sequence can bind to double strand DNA (Xu *et al.*, 1992), as is true with Zfs1 (our unpublished observation). We thus examined whether *zfs1* and *byr3* overlap in function. Although a *zfs1 byr3* double disruptant could hardly

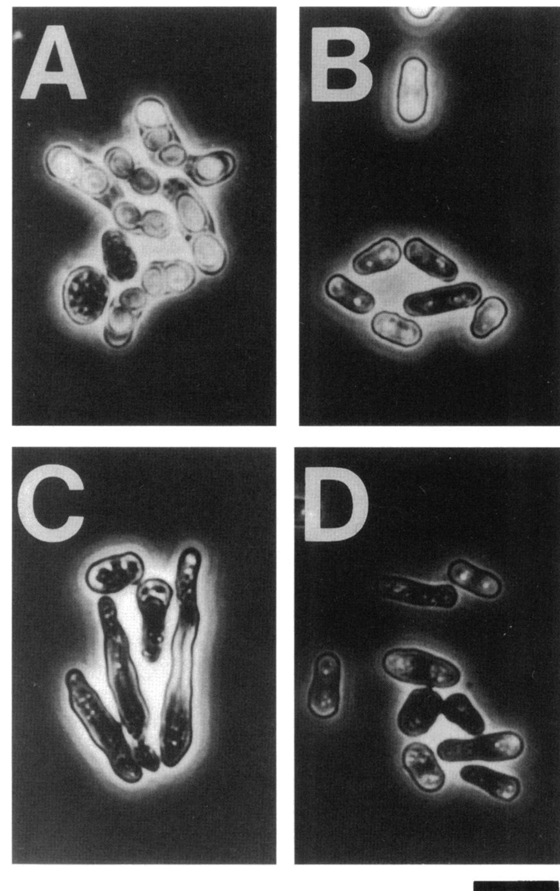

Figure 6. Cell morphology of *ras1* and *zfs1* mutants under nitrogen starvation. (A) JY450 (WT); (B) JZ971 (*zfs1*Δ); (C) JY900 (*ras1^{val17}*); and (D) JX14 (*zfs1*Δ *ras1^{val17}*). Cells were incubated on MEA medium for 2 days. Phase-contrast micrographs are shown. Bar, 10 μm.

conjugate, overexpression of *zfs1* did not suppress *byr3*Δ (JX5) and overexpression of *byr3* did not suppress *zfs1*Δ. It was also noted that overexpression of *zfs1*, unlike that of *byr3*, does not increase the sporulation efficiency in the *ras1*Δ diploid strain. We hence conclude that Zfs1 and Byr3 are unlikely to perform the same function in the mating pheromone signaling pathway.

In summary, the results of the epistasis analyses we carried out did not allow us to pinpoint the step where *zfs1* performs its function in the mating pheromone signaling pathway. However, all the results are consistent with the possibility that *zfs1* is required to enhance the activity of a critical factor in the signaling pathway but is unable to bypass complete loss of the factor even if overexpressed.

Expression of the zfs1 Gene

Figure 7 shows expression of *zfs1* in homothallic haploid cells under nitrogen starvation. The gene was transcribed mainly into mRNA of 3.4 kb in length, suggesting that the transcript has rather long 5′- and 3′-untranslated regions. Expression of *zfs1* was induced about threefold in response to nitrogen starvation. Similar induction was observed in heterothallic cells (our unpublished data). These observations support the view that *zfs1* may play a role in sexual development under nitrogen starvation. At the same time, expression of *zfs1* in cells growing vegetatively (Time 0) should be taken as significant, because *zfs1*-disrupted cells exhibit growth retardation, as stated above.

Cellular Localization of the zfs1 Gene Product

To identify the *zfs1* gene product in fission yeast cell extracts, we prepared rabbit antisera against Zfs1 as described in MATERIALS AND METHODS. We prepared extracts from wild-type *S. pombe* cells with and without pREP1-zfs1, a *zfs1*-overexpressing plasmid. This plasmid carries the *zfs1* ORF under the control of the *nmt1* promoter, which is derepressed in the absence of thiamine and allows considerably strong transcription (Maundrell, 1993). Cells carrying pREP1-zfs1 were grown in either the presence or the absence of thiamine. The cell extracts were run in SDS-PAGE and then analyzed by immunoblotting using affinity-purified anti-Zfs1 antibodies. No band was detectable in the extract prepared from either pREP1-zfs1–transformed cells grown in the presence of thiamine (Figure 8A) or cells carrying no plasmid (our unpublished data). The Zfs1 polypeptide was detected as one band in the extract overexpressing *zfs1* (Figure 8A). The molecular weight of Zfs1 estimated from this analysis was 50 k, which agrees considerably well with the calculated molecular weight of 43,434, although leaving the possibility that Zfs1 is a modified protein.

To determine the cellular localization of the *zfs1* gene product, we performed the following. *S. pombe* cells carrying pREP1-zfs1 were grown in synthetic medium PM and harvested in the log phase. A cell homogenate was prepared and subjected to Percoll gradient centrifugation. After fractionation, each fraction was run in SDS-PAGE and the gel was analyzed by immunoblotting using the affinity-purified anti-Zfs1 antibodies. The results shown in Figure 8B indicate that the immunoreactive material representing

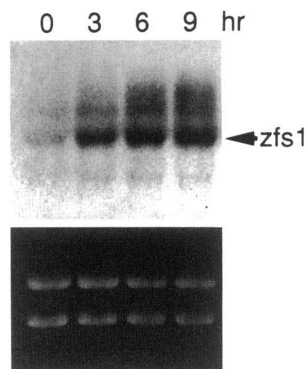

Figure 7. Northern blot analysis of *zfs1* mRNA. RNA was prepared from JY450 (h^{90} wild type) cells growing vegetatively (Time 0) or starved for nitrogen for the time indicated, as in Figure 4. The samples were analyzed by the *zfs1* probe. Approximately equal loading of the RNA samples was confirmed by ethidium bromide staining of rRNA, as displayed in the lower panel.

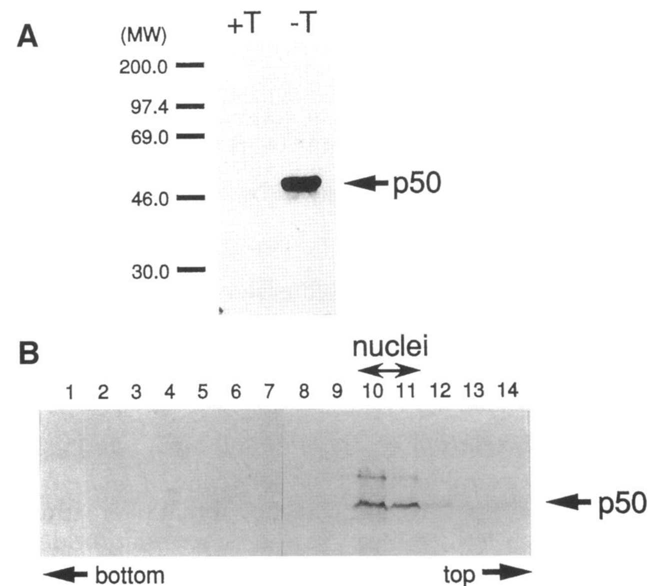

Figure 8. Identification and localization of the *zfs1* protein. (A) Extracts were prepared from *S. pombe* wild-type cells carrying pREP1-zfs1 grown either in the presence of thiamine (+T) or in the absence of thiamine (−T). They were run in SDS-PAGE, blotted to a membrane, and analyzed with anti-Zfs1 antibodies. (B) *S. pombe* wild-type cells carrying pREP1-zfs1 were grown in the absence of thiamine, harvested, and digested with Zymolyase followed by homogenization. The extract was centrifuged on a 15–40% linear Percoll gradient. Fractions were observed microscopically after DAPI staining to examine the presence of nuclei. Each fraction was run in SDS-PAGE and analyzed by immunoblotting as in panel A.

Zfs1 is present almost exclusively in nuclear fractions. Although we cannot completely exclude the possibility that nuclear localization of Zfs1 is an artifactual outcome of overproduction of Zfs1, we can conclude at least that Zfs1 has the ability to migrate into nuclei. We examined a homogenate prepared from the cells subjected to nitrogen starvation, and essentially the same conclusion was drawn with respect to the Zfs1 localization (our unpublished data).

DISCUSSION

Although we originally isolated the *zfs1* gene as a multicopy suppressor of the *pac1*-overexpressing strain, *zfs1* and *pac1* do not appear to function in close association, for the following reasons. The amount of *ste11* mRNA is scarce in the *pac1*-overexpressing strain (Iino *et al.*, 1991), but expression of *ste11* was not greatly reduced in the *zfs1*Δ strain (this study). Introduction of the *zfs1* gene in a multicopy into the *pac1*-overexpressing strain increases the level of *ste11* mRNA only slightly, if at all (our unpublished data). Hence it is unlikely that the primary function of *zfs1* is to counteract *pac1* in the regulation of *ste11* expression. Furthermore, the amount of *zfs1* mRNA was not much affected by overexpression of *pac1* (our unpublished data), excluding the possibility that *zfs1* mRNA is a direct target of Pac1 dsRNase. Thus, we speculate that the observed suppression of the sterility by *zfs1* in the *pac1*-overexpressing strain may be a rather indirect consequence.

Several lines of analysis performed in this study, however, strongly suggest that the function of *zfs1* is required for proper transmission of the mating pheromone signal. Particularly, the phenotype conferred by an activated allele of either *gpa1* or *ras1* was alleviated by disruption of the *zfs1* gene and the deficiency of *zfs1*Δ cells was recovered by overexpression of *gpa1*, *ras1*, *byr1*, or *byr2*. The *zfs1* function is thus likely to be relevant to activation of the MAP kinase cascade, including Byr2, Byr1, and Spk1, which integrates the signals from Gpa1 and Ras1. The observation that the reduced mating ability of the *zfs1*-disruptant could be suppressed by overexpression of either *byr1* or *byr2* appeared to pinpoint its function to the very upstream of the cascade. However, because the *zfs1*-disruptant could also be suppressed by overexpression of either *gpa1* or *ras1*, it is difficult to position the function of Zfs1 directly in the mating pheromone signaling pathway. A simple interpretation of the data would be that *zfs1* is required to enhance the activity of one of the critical factors in the pathway.

Early steps of the mating pheromone signaling are carried out on the plasma membrane. However, our analysis suggested that Zfs1 is likely to locate in nuclei. Our preliminary analysis also suggested that the GST-Zfs1 fusion protein has the ability to bind to DNA (our unpublished data). Therefore, Zfs1 may indirectly influence a critical factor in the signaling pathway, for instance, by regulating expression of another factor that cooperates with the critical factor. The similarity of Zfs1 to mouse Nup475 (this study), as well as the fact that activation of Raf-1 by Ras protein is supported by the 14–3-3 protein in mammalian cells (Freed *et al.*, 1994; Fu *et al.*, 1994; Irie *et al.*, 1994), appears to render this possibility more intriguing.

Byr3 is a zinc-finger protein that appears to affect the Ras1 pathway in *S. pombe* and is able to bind to DNA (Xu *et al.*, 1992). Like Zfs1, however, its function has not been precisely clarified. Although our analysis suggested that these two proteins are unlikely to perform exactly the same function, it will certainly be intriguing to see how these zinc-finger proteins are involved in the promotion of mating pheromone signaling.

Besides Byr3 and Zfs1, several zinc-finger proteins have been shown to function in the sexual development pathway in yeast. *Saccharomyces cerevisiae* Ste5 is a zinc-finger-like protein known to tether three kinases, namely Ste11 (Errede *et al.*, 1993), Ste7 (Teague *et al.*, 1986), and Fus3 (Elion *et al.*, 1990), which constitute the MAP kinase cascade required for mating (Leberer *et al.*, 1993; Choi *et al.*, 1994). It is likely that *S. pombe* has a homologue of Ste5, which will tether Byr2, Byr1, and Spk1, although identification of such a homologue has not yet been made. The *S. pombe rep1* gene is a multicopy suppressor of the *cdc10–129* mutant (Sugiyama *et al.*, 1994), and the product of this gene carries one zinc-finger motif (CCX_1-$CX_{12}HX_3CX_1H$), which is essential for the function of the protein. This motif is a mixture of the two consensus sequences, namely $CX_2CX_{12}HX_3C$ and $CX_1CX_{12}HX_5H$. Disruption of *rep1* has no apparent effect on the mitotic cell cycle and conjugation, but affects initiation of premeiotic DNA synthesis. Similarity or dissimilarity in molecular function between these zinc-finger proteins is largely unclear at present, and further analysis of these proteins is undoubtedly important to obtain profound understanding of the molecular regulation in sexual development.

ACKNOWLEDGMENTS

We thank Drs. C. Shimoda and M. Wigler for the kind gifts of plasmids and strains. This work was supported by Grants-in-Aid from the Ministry of Education, Science, and Culture of Japan. J.K. is a recipient of a JSPS Fellowship for Japanese Junior Scientists.

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