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The Fission Yeast *dis3*$^+$ Gene Encodes a 110-kDa Essential Protein Implicated in Mitotic Control

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The fission yeast mutant *dis3-54* is defective in mitosis and fails in chromosome disjunction. Its phenotype is similar to that of *dis2-11*, a mutant with a mutation in the type 1 protein phosphatase gene. We cloned the *dis3*$^+$ gene by transformation. Nucleotide sequencing predicts a coding region of 970 amino acids interrupted by a 164-bp intron at the 65th codon. The predicted dis3$^+$ protein shares a weak but significant similarity with the budding yeast *SSD1* or *SRK1* gene product, the gene for which is a suppressor for the absence of a protein phosphatase *SIT4* gene or the *BCY1* regulatory subunit of cyclic AMP-dependent protein kinase. Anti-dis3 antibodies recognized the 110-kDa *dis3*$^+$ gene product, which is part of a 250- to 350-kDa oligomer and is enriched in the nucleus. The cellular localization of the dis3$^+$ protein is reminiscent of that of the dis2$^+$ protein, but these two proteins do not form a complex. A type 1 protein phosphatase activity in the *dis3-54* mutant extracts is apparently not affected. The *dis3*$^+$ gene is essential for growth; gene disruptant cells do not germinate and fail in cell division. Increased *dis3*$^+$ gene dosage reverses the Ts$^+$ phenotype of a *cdc25 wee1* strain, as does increased type 1 protein phosphatase gene dosage. Double mutant *dis3 dis2* is lethal even at the permissive temperature, suggesting that the *dis2*$^+$ and *dis3*$^+$ genes may be functionally overlapped. The role of the *dis3*$^+$ gene product in mitosis is unknown, but this gene product may be directly or indirectly involved in the regulation of mitosis.

Protein phosphorylation and dephosphorylation are important posttranslational modifications essential for a variety of biological systems. In mitotic cell cycle control, cdc2$^+$ protein kinase plays a pivotal role as a component of maturation/M-phase promoting factor (21); its phosphorylation state changes during the cell cycle, and its activity is regulated by dephosphorylation (9). In contrast to the identification of an ever-increasing number of protein kinases (15), protein dephosphorylation seems to be carried out by a limited number of protein phosphatases (PPases) (6); in mammals, there are generally four serine- and threonine-specific PPases, that is, types 1 (PP1), 2A (PP2A), 2B (PP2B), and 2C (PP2C). An apparent paradox is how PPases can counteract the diversity of protein kinases. One possibility is that there may be many more PPases awaiting discovery. Alternatively, the diversity of PPases may be based on the presence of specific modulators, inhibitors, and activators (16, 25, 30, 35).

The essential roles of PPases in the mitotic cell division cycle have only recently been established. Mutations in the PP1 catalytic subunit were identified in the filamentous fungus *Aspergillus nidulans* (*BimG* mutant [8]) and the fission yeast *Schizosaccharomyces pombe* (*dis2* mutant [24]). Failure in chromosome disjunction was the common phenotype of fission yeast, filamentous fungus, and fruit fly PP1 mutants (2, 8, 24). In *S. pombe*, there are two PP1 catalytic subunit genes, namely, *dis2*$^+$ and *sds21*$^+$. Results of gene disruption experiments showed that the single-gene disruptants (Δ*dis2* and Δ*sds21*) were viable whereas the double-gene disruptant (Δ*dis2* Δ*sds21*) was not, indicating that these two genes share an essential function (24). The cold-sensitive (cs) *dis2-11* mutation appeared to be semidominant (19, 24). There are also two PP2A genes (*ppa1*$^+$ and *ppa2*$^+$) in fission yeast. Again, single-gene disruptants were viable but the double-gene disruptant was not. The semidominant cs PP2A mutant was found to display a phenotype quite distinct from that of the PP1 mutant (19).

Two other classes of *dis* mutants (*dis1* and *dis3*) which show a defective phenotype similar to that of the *dis2-11* mutant are known (23). All of them were identified as cs mutants in which, at the restrictive temperature (20°C), mitotic chromosome disjunction is blocked. These cs mutants enter mitosis at 20°C with normal timing but lose viability during mitosis. There is extensive phenotypic similarity among the different *dis* mutants. All of the mutants are caffeine hypersensitive. They lose artificially constructed minichromosomes at high rates. Despite these similarities, however, genetic crossing indicates that they represent three different genetic loci. cs *dis2-11* cells indeed lack PP1 enzyme activity at 20°C (19). However, no attempt has been made to assay PPase activity in *dis1* and *dis3* mutant extracts.

We addressed the question of whether the *dis1*$^+$ and *dis3*$^+$ genes are functionally related to the *dis2*$^+$ gene. Do they encode other phosphatases or modulators, inhibitors, activators, or substrates of phosphatase? Alternatively, is the *dis* phenotype similarity coincidental, not necessarily reflecting a functional similarity of the gene products? The fact that a high *dis2*$^+$ gene dosage extragenically complements *dis1* mutants (24) favors the possibility that the *dis1*$^+$ gene product is implicated in protein dephosphorylation. However, the nature of the *dis1*$^+$ gene product is unknown. On the other hand, no functional link except the phenotypic similarity is known between the *dis3*$^+$ and *dis2*$^+$ genes. In the present study, we have investigated the gene function of *dis3*$^+$ and examined whether the *dis3*$^+$ gene product is implicated in protein dephosphorylation. To this end, we

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TABLE 1. Synthetic lethality of *dis3* and *dis2* mutants

Cross[a]	Colony formation	Phenotype of double mutants
dis3-54 × *dis2-11*	−	Nonviable (no germination)
dis3-54 × *dis1-288*	−	Nonviable (two to three divisions)
dis3-54 × *dis2*::*ura4*$^+$	−	Nonviable (germination but no division)
dis3-54 × *sds21*::*LEU2*	cs	Viable (cs)

[a] The genotypes of the strains used were as follows: *dis3-54*, h^+ *leu1 his2 dis3-54* or h^- *leu1 dis3-54*; *dis2-11*, h^- *leu1 dis2-11*; *dis2*::*ura4*$^+$, h^- *leu1 ura4 dis2*::*ura4*$^+$; and *sds21*::*LEU2*, h^+ *leu1 his2 sds21*::*LEU2*. The gene-disrupted strains *dis2*::*ura4*$^+$ and *sds21*::*LEU2* (24) are viable; cell lethality is caused only when both the *dis2* and the *sds21* genes are disrupted (24).

isolated the *dis3*$^+$ gene, examined by gene disruption whether it is essential, and identified and characterized the *dis3*$^+$ gene product with anti-dis3 antibodies. We also investigated the *dis3* mutant with regard to PPase activity, cell cycle arrest, and the relationship to cell cycle-controlling genes.

MATERIALS AND METHODS

Strains, media, and chemicals. Haploid *S. pombe* strains were used. The genotypes of the strains used for gene disruption are indicated in Table 1. Rich (YPD), minimal (EMM2), nitrogen source-deprived (EMM2−N), and sporulation (SPA) media were used (11). TEG buffer contained 50 mM Tris HCl (pH 7.5), 1 mM EDTA, 10% glycerol, 1 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride.

Transformation, integration, and gene disruption. The lithium acetate method (17) was used for transformation of *S. pombe* cells. Integration of cloned sequences into the chromosome was performed by homologous recombination (27). For disruption of the *dis3*$^+$ gene, plasmid pCD140 was constructed, linearly cleaved, and used for transformation. Stable transformants were analyzed by tetrad analysis and Southern hybridization.

Blotting and nucleotide sequence determination. Standard procedures for Southern and Northern (RNA) blotting (20) were used. Immunoblotting was performed by electrophoretically transferring proteins to nitrocellulose after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (34). Nucleotide sequences were determined by the dideoxy method with pUC plasmids (38).

Construction of the fusion protein and preparation of antiserum. Plasmid pAR3039 (31) containing the T7 promoter was used for expression of the *dis3*$^+$ coding region in bacterial strain BL21. The 2.2-kb *Bgl*II fragment containing a part of the *dis3*$^+$ gene was ligated to the plasmid, and the resulting plasmid, pCD141, caused the synthesis of a large quantity of a 70-kDa protein in bacterial cells induced by isopropyl-β-D-thiogalactopyranoside. This 70-kDa protein was purified by previously described procedures (26), and 200 μg of polypeptide was injected into rabbits every 2 weeks for 10 weeks.

Immunofluorescence microscopy and FACScan analysis. Previously described procedures for *S. pombe* immunofluorescence microscopy were used (10, 18). The cell wall was digested with 0.2 mg of Zymolyase per ml and partially purified Novo Biolabs mutanase. The second antibody was a rhodamine-conjugated goat anti-rabbit immunoglobulin G antibody. A Becton-Dickinson FACScan was used to monitor the cellular DNA contents. The procedures used were similar to those described previously (7). Cells (1×10^7 to 5×10^7) were collected, washed twice with 1 ml of distilled water, and resuspended in 3 ml of distilled water. Ethanol (7 ml) was added with vigorous agitation, and the cells were stored at 4°C for more than 12 h. After one wash with 5 ml of 50 mM sodium citrate (pH 7.0) and resuspension in 1 ml of the same buffer, RNase A (Sigma; preboiled for 15 min) was added to a final concentration of 0.5 mg/ml. Following 2 h of incubation at 37°C, propidium iodide (Sigma) was added (final concentration, 12.5 μg/ml) and the resulting stained cell suspensions were analyzed.

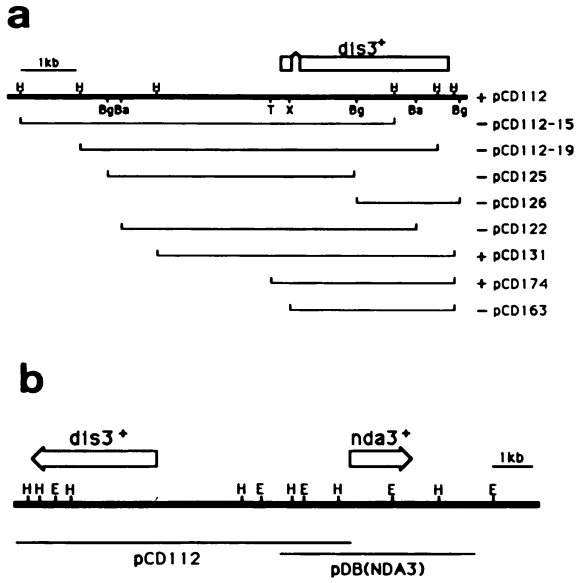


FIG. 1. (a) Subcloning of the genomic DNA sequence that complements the cs *dis3* mutation (+ indicates complementation). H, *Hin*dIII; Ba, *Bam*HI; Bg, *Bgl*II; T, *Tth*111I; X, *Xho*I. The box indicates the coding region. (b) Linkage between the *dis3*$^+$ and *nda3*$^+$ genes. Plasmids pCD112 and pDB(NDA3) contain the overlapping region. H, *Hin*dIII; E, *Eco*RI.

GenBank-EMBL accession number. The GenBank-EMBL accession number for the sequence of the *dis3*$^+$ gene is M74094.

RESULTS

Isolation of the *dis3*$^+$ gene by complementation. A fission yeast genomic DNA fragment (pCD112; Fig. 1a) that complemented cs *dis3-54* was isolated. This fragment also complemented the caffeine hypersensitivity of the mutant. Subsequent subcloning indicated that a 3.3-kb fragment was the minimal requirement for the complementation of cs *dis3-54* (pCD174; Fig. 1a). Multicopy plasmid pCD112 failed to complement other *dis* gene mutants, namely, *dis1-288* and *dis2-11*. Genomic Southern hybridization (50°C, 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) blots probed with the *dis3*$^+$ coding fragment in pCD174 showed only an intense self-hybridizing band (data not shown), suggesting that the *dis3*$^+$ gene is unique in the genome. Attempts to clone a budding yeast homolog by hybridization were not successful.

The 1.4-kb *Hin*dIII fragment in pCD112 was integrated into the chromosome by homologous recombination with chromosome integration vector pYC20 (5). Tetrad analysis

showed that the integrated locus was tightly linked to the *dis3* locus (parental ditype:nonparental ditype:tetratype, 44:0:0). Therefore, the isolated sequence should be derived from the *dis3*$^+$ gene.

A previous genetic analysis (23) showed that the *dis3* locus is very close to the *nda3* locus (map distance, ~1 centimorgan). Indeed, we found that the sequence in pCD112 overlapped that in pDB(NDA3), which carried the *nda3*$^+$ gene (14) (Fig. 1b). The distance between *dis3*$^+$ and *nda3*$^+$ is 5 kb, consistent with the genetic distance. Three-factor crosses showed that the *dis3* locus is centromere distal relative to the *nda3* locus (data not shown).

The nucleotide sequence of *dis3*$^+$ predicts a 110-kDa protein. The nucleotide sequence of pCD174 was determined by the dideoxy method (Fig. 2). It predicts a hypothetical dis3$^+$ polypeptide with a 164-bp putative intron (the consensus sequences are underlined) at the 65th codon, a total of 970 amino acids, and a predicted molecular mass of 110 kDa (pI, 6.5). Northern hybridization blots of *S. pombe* poly(A)$^+$ mRNA probed with the coding fragment showed a 2.8-kb band (data not shown), consistent with the size of the predicted polypeptide.

A computer search of the EMBL database did not reveal any homology to known protein sequences. However, as shown in Fig. 3, weak but significant homology to the *Saccharomyces cerevisiae SRK1* gene (36) and *SSD1* gene (32) was found. There are 23.5% identical and 37% similar conserved amino acids in 523 amino acids. The highest similarity is found in the COOH domains of the dis3$^+$ and SRK1 or SSD1 proteins (Fig. 3); 22 residues are identical (69% identity) in the 32-amino-acid stretch from positions 796 to 827 of the dis3$^+$ protein. The *SSD1* gene can suppress the lethality attributable to the deletion of an essential PPase gene, *SIT4* (1, 32). The *SRK1* gene (36) was identified as a suppressor of a mutation in *BCY1*, the gene for the regulatory subunit of cyclic AMP-dependent protein kinase (29, 33, 37). The *SRK1* and *SSD1* genes were found to be identical. The SRK1 or SSD1 protein is 280 amino acids larger than the dis3$^+$ protein.

To examine whether *dis3*$^+$ and *SRK1* have identical functions, we introduced a plasmid carrying the *SRK1* gene (pW31, a gift from K. Tatchell) into cs *dis3* mutant cells by transformation and tested it for complementation. However, no Cs$^+$ transformant was obtained. This result, together with the low sequence similarity and size difference, suggested that the *dis3*$^+$ and *SRK1* or *SSD1* genes are not true homologs. Because the *dis3*$^+$ gene seems to contain an intron which is not spliced in budding yeast cells, transformation of an *srk1* or an *ssd1* mutant by the *dis3*$^+$ gene has not been done.

The *dis3*$^+$ gene is essential for growth. One-step gene disruption was carried out as shown in Fig. 4. Tetrad analysis indicated that fewer than two spores were viable, and all the viable spores were Leu$^-$. Therefore, a *dis3* disruption is lethal; *dis3*$^+$ is an essential gene. Genomic Southern hybridization of heterozygous diploids (+/Δ*dis3*; Fig. 4) and a wild-type diploid (+/+) produced hybridizing bands of the expected sizes.

None of the *dis3*::*LEU2*-disrupted spores germinated on YPD plates, indicating that the *dis3*$^+$ gene is essential for germination. The *dis3*-disrupted heterozygous diploid cells were transformed by plasmid pCD131 and sporulated. The resulting plasmid-carrying spores were germinated, grown in minimal EMM2 medium, and transferred to complete medium at a restrictive temperature for 20 h. Cells which have lost the plasmid should stop dividing and reveal the defective phenotype. However, arrested cells proved difficult to distinguish from plasmid-harboring dividing cells by fluorescence microscopy with 4′,6-diamidino-2-phenylindole (DAPI) stain. We did not see cells with a mitotic arrest phenotype reminiscent of that of the cs *dis3-54* mutants (data not shown). The cell cycle phenotype of cs *dis3-54* might be allele specific.

Identification of the *dis3*$^+$ gene product. To identify the *dis3*$^+$ gene product, we raised rabbit antiserum against the dis3$^+$ protein as described in Materials and Methods. The antiserum was affinity purified with the fusion polypeptide. *S. pombe* protein extracts were run in SDS-PAGE and immunoblotted. A protein band with a molecular mass of 110 kDa was obtained; this band was not seen with preimmune serum (data not shown). Introduction of the high-copy-number *dis3*$^+$ plasmid (pCD174) into *S. pombe* wild-type cells failed to increase significantly the level of this 110-kDa polypeptide (data not shown), so the experiment described below was required to obtain definitive evidence to assign the 110-kDa polypeptide as the *dis3*$^+$ gene product.

Eight truncated *dis3*$^+$ genes (172A to 172H) were made by a series of deletions from the NH_2 or COOH termini (Fig. 5a). 172A lacks the first 74-amino-acid portion, whereas 172F terminates 147 residues before the wild-type termination codon. These truncated genes were ligated with the alcohol dehydrogenase gene promoter sequence (28), and the resulting plasmids were introduced into cs *dis3-54* mutants. Immunoblots of some of these strains (Fig. 5b) showed polypeptide bands with the molecular masses expected for these truncated genes. The predicted mass of the 172A gene product was consistent with the presence of a putative intron. (Polypeptides encoded by 172G and 172H and consisting of the NH_2 domain of the dis3$^+$ protein were not detected because the anti-dis3 antibodies were raised against the COOH domain). These results proved that the bands detected by anti-dis3 antibodies represented the *dis3*$^+$ gene product in *S. pombe* extracts. None of these truncated genes, except for 172A, was able to complement cs *dis3-54* mutants (although the colonies formed with 172A were small). Hence, the NH_2-terminal 74 amino acids appear not to be essential.

The *dis3*$^+$ gene product is enriched in the nucleus. Immunoblotting of salt-extracted fractions indicated that the dis3$^+$ protein was soluble in TEG buffer (see Materials and Methods) containing 0 to 0.1 M NaCl. Most of the dis3$^+$ protein was found in the supernatant fraction of glass bead-broken extracts after centrifugation at 50,000 × *g* for 20 min. We performed Percoll gradient centrifugation of cellular homogenates; this process fractionates the nuclei from the cytosol (11, 13). The dis3$^+$ protein was present in both the higher cytosol and the lower nuclear fractions (data not shown).

Immunofluorescence microscopy of growing wild-type cells with affinity-purified anti-dis3 antibodies revealed intense nuclear immunofluorescence (Fig. 6a). Additional granular fluorescence was also found in the cytoplasm. The dis3$^+$ protein exists in both the nucleus and the cytoplasm but is enriched in the nucleus. A comparison of DAPI-stained DNA and anti-dis3 antibody immunofluorescence showed that chromosomes were weakly stained, whereas immunofluorescence in the nonchromosomal domain was intense (Fig. 6a). These results were clearly demonstrated in the dividing nuclei. The level of nuclear immunofluorescence did not appear to be altered during the cell cycle. This anti-dis3 antibody immunofluorescence pattern was similar to that obtained with anti-dis2 antibodies (24).

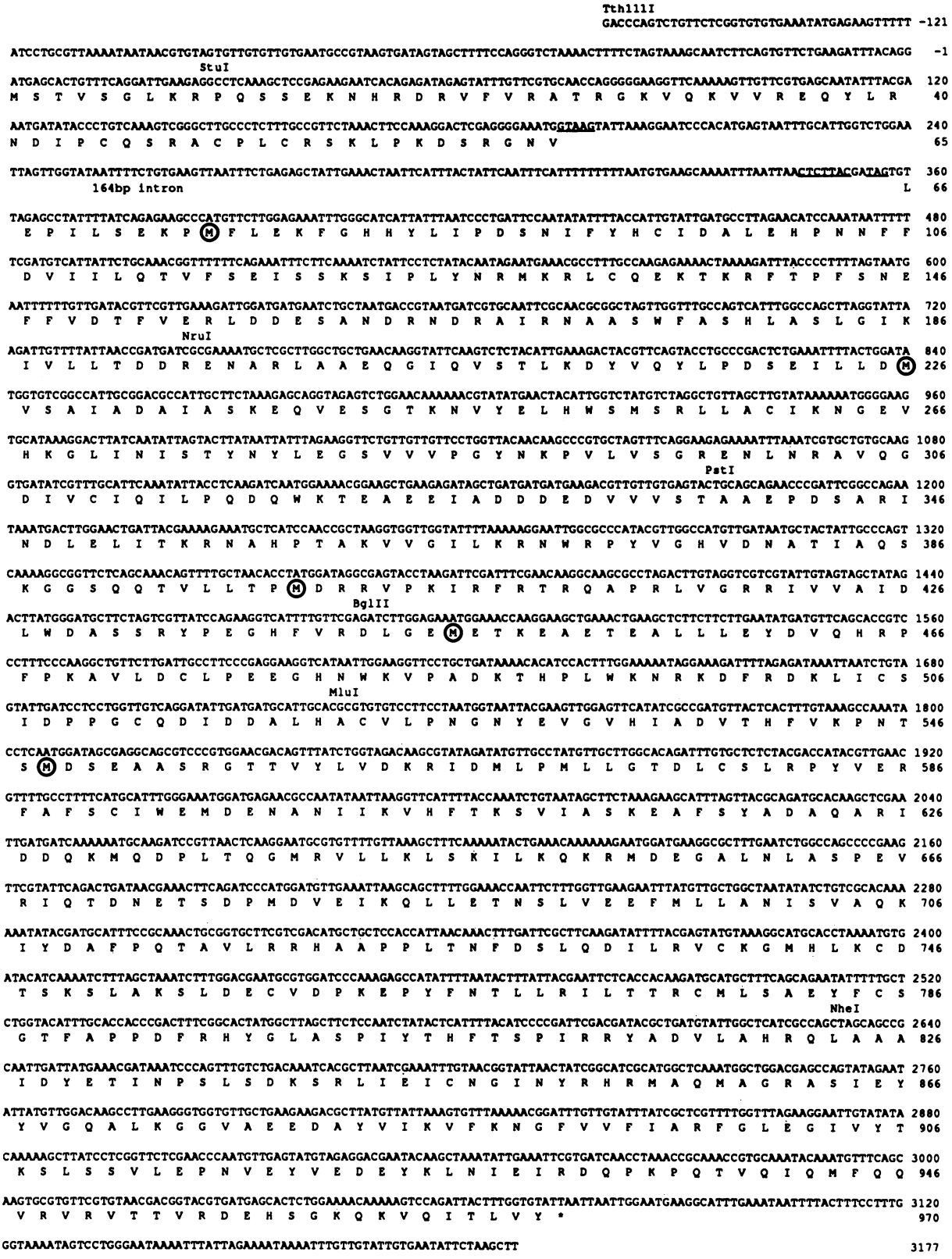
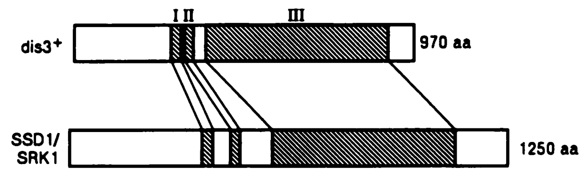


FIG. 2. Nucleotide sequence of the *dis3*$^+$ gene and predicted amino acid sequence. The putative intron consensus sequences are underlined. The restriction sites used for construction of the truncated genes are indicated, and the circled Ms (methionines) represent the putative initiation codons of the truncated genes.

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I dis3+ 289 NKPVLVSGRENLNRAVQGDIVCIQILPQD
SSD1/   383 DADIYICGSKDRNRALEGDLVAVELLVVD
SRK1

II      332 DVVVSTAAEPDSARINDLELITKRNA
        456 NNFLSSPSSSDSLSKDDLSVRRKRSS

III     390 SQQTVLLTPMDRRVPKIRFRTRQAPRLVGRRIVVAIDLWDASSRYPEGHFVRDLGEMETK
        587 TDKKVPLIAIPTELAPKDF-VENADKYSEKLFVASIKRWPITSLHPFGILVSELGDIHDP

        450 EAETEALLLEYDVQHRPFPKAVLDCLPEEGHNWKVPADKTHPLWKNRKDFRDKLICSIDP
        646 DTEIDSILRDNNFLSNEYLDQ--KNPQKEKPSFQPLPLTAESL-EYRRNFTDTNEYNIFA

        510 PGCQD-IDD-ALHACVLPNGNYEVGVHIADVTHFVKPNTSMDSEAASRGTTVYLVDKRID
        703 ISELGWVSEFALHVRNNGNGTLELGCHVVDVTSHIEEGSSVDRRARKRSSAVFMPQKLVN

        568 MLPMLLGTDLCSLRPYVERFAFSCIWEMDENANIIKVHF-TKSVIASKEAFSYADAQARI
        763 LLPQSFNDEL-SLAPGKESATLSVVYTLDSSTLRIKSTWVGESTISPSNILSLEQLDEKL

        627 DDQKMQDPLTQGMRVLLKLSKILKQKRMDEGALNLASPEVRIQTDNETSDPMDVEIKQLL
        822 ST----GSPTSYLSTVQEIARSFYARRINDPEATLLPTLSLLESLDDEKVKVDLNILDRT

        687 ETNSLVEEFMLLANISVAQKIYDAFPQTAVLRRHAAPPLTNFDSLQDILRVCKGMHLKCD
        878 LGFVVINEIKRKVNSTVAEKIYTKLGDLALLRRQMQPIATKMASFRKKIQNF-GYNFDTN

        747 TSKSLAKSLDECVDPKEPYFNTLLRILTTRCMLSAEYFCSGTFAPPDFRHYGLASPIYTH
        937 TADELIKGV---LKIKDDDVRVGIEILLFKTMPRARYFIAGKVDPDQYGHYALNLPIYTH

        807 FTSPIRRYADVLAHRQLAAAIDYETINPSLSDKSRLIEICN---GINYRHRMAQMAGRAS
        994 FTAPMRRYADHVVHRQLKAVIHDTPYTEDMEALKITSEYCNFKKDCAYQAQEQAIHLLLC

        864 IEYYVGQALKGGVAEEDAYVIKVFKNGFVVFIARFGLEGIVYTKSLSSV   912
       1054 KTINDMGNTTGQLLTMAT-VLQVYESSFDVFIPEFGIEKRVHGDQLPLI  1101
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FIG. 3. Amino acid (aa) sequence homology between the fission yeast *dis3*+ and the budding yeast *SRK1* or *SSD1* gene products. Similar domains I, II, and III in the proteins are schematically shown. Identical and conserved amino acids are indicated by double and single dots, respectively.

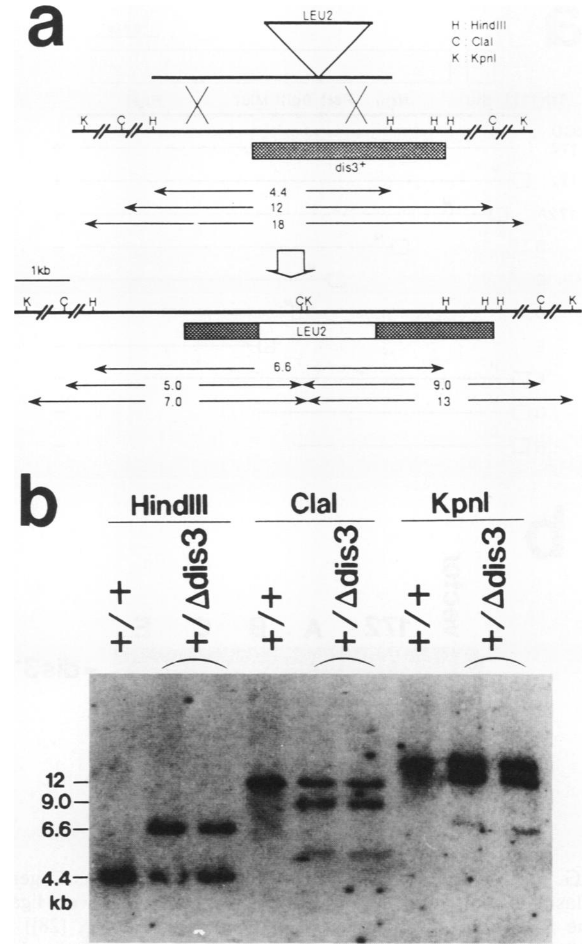

FIG. 4. Disruption of the *dis3* gene. (a) Construction of the disrupted *dis3*::*LEU2* gene. The 4.4-kb *Hin*dIII fragment was inserted at the *Bgl*II site with the *S. cerevisiae LEU2* gene. Linear DNA containing the disrupted *dis3* gene (*dis3*::*LEU2*) was used for transformation of *S. pombe leu1*− diploid cells (strain CM3/CM7; *h*−/*h*+ +/*his2 leu1*/*leu1 ura4*/*ura4 ade6-M210*/*ade6-M216*), and the resulting heterozygous diploids containing the integrated *dis3*::*LEU2* were sporulated by nitrogen starvation. The expected lengths of restriction fragments with or without the gene disruption are shown in kilobases. The 4.4-kb *Hin*dIII fragment was used as a probe. (b) Genomic Southern hybridization of the wild type (+/+) and two *Leu*+ integrants (+/Δ*dis3*) probed with a 1.1-kb *Bgl*II-*Bam*HI fragment. Genomic DNAs were digested with *Hin*dIII, *Cla*I, or *Kpn*I and run in an agarose gel. The sizes of fragments are indicated in kilobases.

An immunofluorescence micrograph of *nuc2* mutant cells revealing condensed chromosomes (11, 12) is shown in Fig. 6b. Punctate staining of chromosomes with anti-dis3 antibodies was observed in addition to nonchromosomal staining.

The *dis3*+ gene product exists in an oligomeric form. In gel filtration of wild-type *S. pombe* extracts, the dis3+ polypeptide eluted in the 250- to 350-kDa range (Fig. 7a, middle panel). This result indicates that the dis3+ protein does not exist in a monomeric form in its native state. The elution characteristics of the dis3+ protein partly match those of the dis2+ protein (Fig. 7a, lower panel), as determined by immunoblotting with anti-dis2 antibodies. PP1, assayed with ^{32}P-labeled phosphorylase as a substrate in the presence of 10 nM okadaic acid, which inhibits PP2A but not PP1, eluted in the ~80-kDa range (Fig. 7a, upper panel). Therefore, the dis3+ protein is probably not a component of PP1, at least when phosphorylase is used as a substrate.

Consistently, immunoprecipitates obtained with anti-dis2 antibodies (indicated by anti dis2 ppt in Fig. 7b) contained dis2+ protein, which was detected by immunoblotting with anti-dis2 antibodies (left side of lower panel), but did not contain dis3+ protein (left side of upper panel). Similarly, immunoprecipitates obtained with anti-dis3 antibodies (indicated by anti dis3 ppt in Fig. 7b) contained dis3+ protein (upper panel) but not dis2+ protein (lower panel). Thus, complex formation between the dis2+ and dis3+ proteins is unlikely.

Double mutants involving *dis3*, *dis1*, and *dis2* are nonviable. Because the phenotype of *dis3-54* is similar to that of *dis2-11* and *dis1-288*, we tried to construct double mutants by crossing. If two recombined genes share a similar, indispensable function, then a double mutation may cause cell death, even at the permissive temperature. Tetrads resulting from the crosses listed in Table 1 were dissected.

Colony-forming double mutants were not obtained from these crosses, except for *dis3*cs *sds21*null, which could grow at the permissive temperature. Double-mutant spores derived from the other three crosses, namely, *dis3*cs *dis2*cs, *dis3*cs *dis1*cs, and *dis3*cs *dis2*null, could not form colonies. Similarly, crossing of cs *dis1* and cs *dis2* did not yield a viable double mutant. Thus, the synthetic lethality obtained

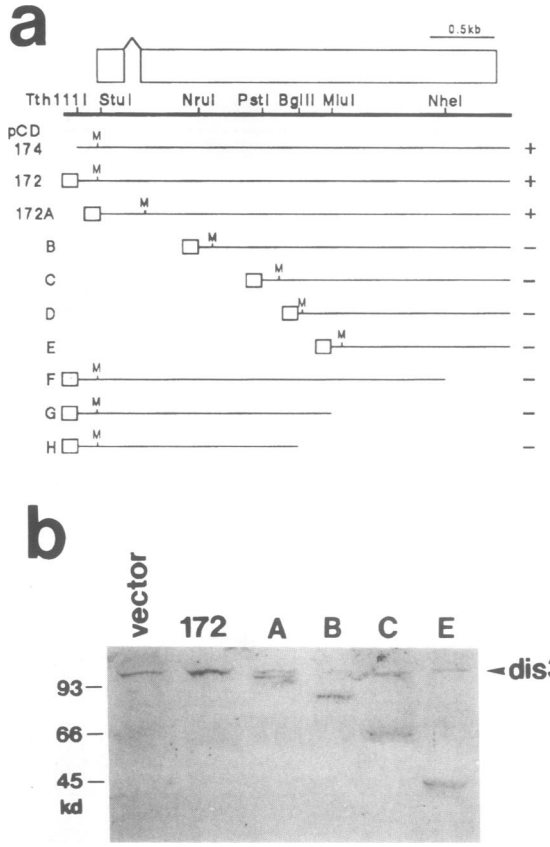

FIG. 5. Construction and expression of truncated *dis3*$^+$ genes. (a) Plasmids containing different parts of the *dis3*$^+$ gene were ligated to the *S. pombe* ADH promoter (indicated by the box [28]) and transformed into cs *dis3* mutant cells. M, putative initiation codons. Complementation is indicated on the right. The wild-type gene and, to a lesser degree, a truncated gene (172A) lacking 74 NH_2-terminal residues could complement cs *dis3*. (b) Immunoblotting of wild-type and transformant extracts with anti-dis3 antibodies. Polypeptides of expected molecular masses were detected.

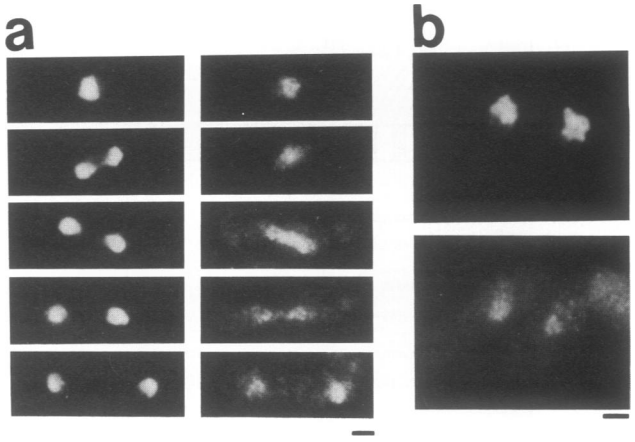

FIG. 6. Localization of the *dis3*$^+$ gene product by immunofluorescence microscopy. *S. pombe* cells were fixed and prepared for immunofluorescence microscopy with anti-dis3 antibodies. (a) Wild-type cells. Left, DAPI stain for chromosomal DNA. Right, anti-dis3 antibody stain. (b) ts *nuc2* mutant cells containing mitotic condensed chromosomes. Top, DAPI stain. Bottom, anti-dis3 antibody stain. Bars, 2 μm.

by combining these three *dis* mutations indicates functional similarity among the *dis*$^+$ genes. The viability of *dis3*^{cs} *sds21*^{null} at the permissive temperature may be explained by the fact that the *sds21*$^+$ gene encodes a minor PP1 (19, 24).

High-dosage expression of *dis3*$^+$ reverses the *wee1 cdc25* phenotype. Other similarities between the *dis2*$^+$ and *dis3*$^+$ genes come from their interrelationship with the cell cycle regulator genes. The *bws1*$^+$ gene (identical to *dis2*$^+$) was cloned by its ability to reverse the Ts$^+$ phenotype of the temperature-sensitive (ts) double mutant *cdc25-22 wee1-50* (4). *cdc25*$^+$ is a mitosis inducer, while *wee1*$^+$ is a mitosis inhibitor (21). To examine whether the high-dosage *dis3*$^+$ gene has the same ability, we used a multicopy plasmid or a high-expression plasmid with the fission yeast ADH promoter (28) and the *dis3*$^+$ gene for transformation. The ADH-*dis3*$^+$ gene could reverse the phenotype of *cdc25 wee1* (Table 2). The multicopy plasmid carrying the *sds21*$^+$ gene coding for a PP1 gene also could reverse the phenotype. However, the multicopy plasmid carrying the *ppa2*$^+$ gene (a PPA2 gene [19]) could not.

PPase activity in *dis3* mutant extracts. PP1 activity in *dis3* mutant extracts was assayed with ^{32}P-labeled phosphorylase as the substrate. Cell extracts were fractionated by DEAE-Sepharose chromatography as described previously (19). Two peaks, A and B, were obtained for phosphorylase phosphatase activity (Fig. 8). Peaks A and B correspond to PP1 and PP2A activities, respectively (19). In *dis2* and *ppa2* mutant extracts, these PP1 and PP2A peaks, respectively, were greatly reduced (19). In *dis3* mutant extracts prepared at the restrictive temperature (Fig. 8a), PP1 activity was not reduced. In *dis3* mutant cells carrying a plasmid with the *dis3*$^+$ gene, a similar elution profile was obtained (Fig. 8b). Thus, although the phenotype of *dis3* mutants is similar to that of *dis2* mutants, the levels of PP1 activity in *dis3* mutant extracts monitored as phosphorylase phosphatase were not affected.

The *dis3* and *dis2* mutants can transit G_1/S. We addressed the question of whether the cs *dis3-54* and *dis2-11* mutants can transit the G_1/S boundary at a restrictive temperature (20°C). This point had not been established previously, as the duration of the G_1 phase is negligible in exponentially growing fission yeast cells. To this end, cells growing at a permissive temperature (33°C) were temporarily starved in nitrogen-deficient medium (EMM2-N), in which cells enter a G_1-like phase (22); cells resume growth upon readdition of a nitrogen source.

dis3-54, *dis2-11*, and wild-type cells were grown in complete medium EMM2 at 33°C, transferred to nitrogen source-deficient medium EMM2-N for 7 h, and returned to complete medium EMM2 again at either the restrictive or the permissive temperature. Cellular DNA contents were analyzed with a Becton-Dickinson FACScan (see Materials and Methods). All the cultures clearly showed a high accumulation of cells with 1c DNA content at 7 h after deprivation of the nitrogen source (Fig. 9a). These cells appeared to move across the G_1/S phases after readdition of the nitrogen source at 20 as well as 33°C. Therefore, *dis3-54* and *dis2-11* cells are not defective in transit of the G_1/S boundary. Consistent with this idea, cells with 1c DNA content were not observed in a *dis3-54* culture grown asynchronously at the permissive temperature and transferred to nonpermissive conditions for 10 h (Fig. 9b). If *dis3-54* cells were blocked at two points, G_1/S and G_2/M, two peaks corre-

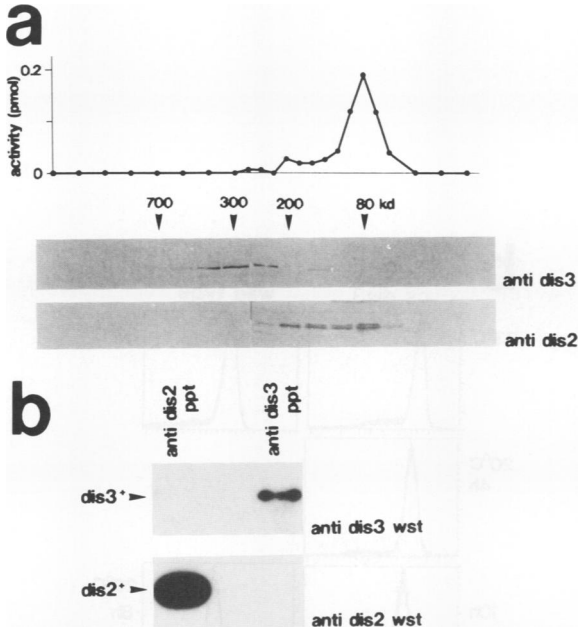

FIG. 7. (a) Gel filtration chromatography of dis3$^+$ and dis2$^+$ proteins. Wild-type cell extracts were prepared and fractionated by gel filtration chromatography with Superose 6 (Pharmacia). Each fraction was assayed by immunoblotting with anti-dis3 and anti-dis2 antibodies. The dis3$^+$ protein was eluted in the range of 250 to 350 kDa, and the dis2$^+$ protein was eluted in the range of 80 to 200 kDa. A PP1 was assayed with ^{32}P-labeled phosphorylase as the substrate in the presence of 10 nM okadaic acid. PP2A was inhibited by 10 nM okadaic acid. (b) Immunoblotting of immunoprecipitates by anti-dis3 and anti-dis2 antibodies. First, the wild-type cell extracts were immunoprecipitated by anti-dis3 or anti-dis2 antibodies. Then, the immunoprecipitates were run in SDS-PAGE and immunoblotted by anti-dis3 or anti-dis2 antibodies (indicated by anti dis3 wst and anti dis2 wst, respectively). The immunoprecipitates obtained with anti-dis2 antibodies did not contain dis3$^+$ protein, while those obtained with anti-dis3 antibodies did not contain dis2$^+$ protein.

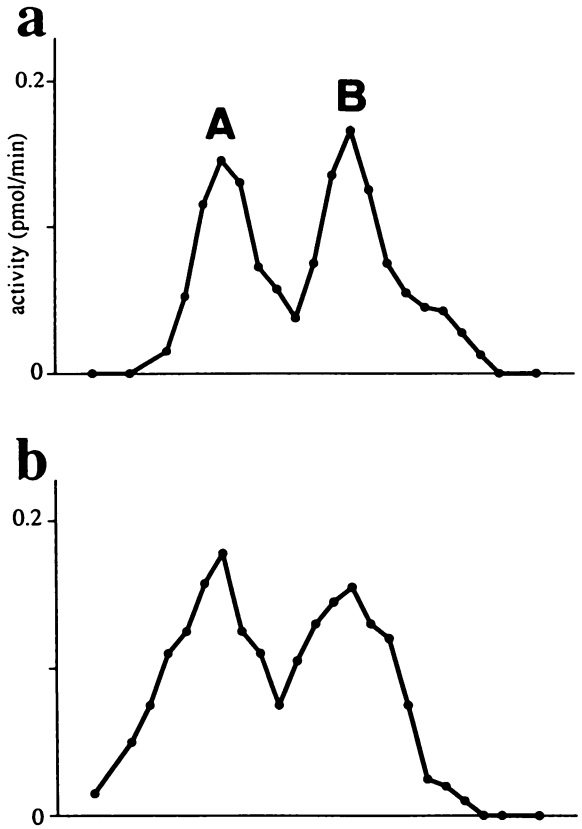

FIG. 8. Assay of PPase activity in *dis3* mutant extracts with ^{32}P-labeled phosphorylase as the substrate. The mutant extracts were fractionated with DEAE-Sepharose, and two activity peaks, A and B (eluting at 0.17 and 0.24 M NaCl), corresponding to PP1 and PP2A, respectively, were obtained (19). The extracts used were obtained from *dis3* mutant cells incubated at the restrictive temperature (20°C) for 10 h (a) and from *dis3* mutant cells carrying plasmids with the wild-type *dis3*$^+$ gene and grown at 20°C (b).

sponding to different DNA contents should have been present. On the basis of these results, we conclude that *dis3-54* and *dis2-11* mutants are blocked only in mitosis. These results, however, do not exclude the possibility that the *dis2*$^+$ and *dis3*$^+$ genes function in G_1/S, because the lethal phenotype of cs *dis2* and *dis3* mutants is allele dependent.

TABLE 2. Reversal of the *cdc25 wee1* double-mutant phenotype by the *dis3*$^+$ gene with the ADH promoter[a]

Plasmid	Gene	Colony formation
Multicopy	None	Ts$^+$
Multicopy	*dis2*$^+$	Ts$^-$
ADH	*dis3*$^+$	Ts$^-$
Multicopy	*sds21*$^+$	Ts$^-$
Multicopy	*ppa2*$^+$	Ts$^+$
Multicopy	*sds22*$^+$	Ts$^+$
Multicopy	*sds23*$^+$	Ts$^+$

[a] *S. pombe* double mutant *cdc25 wee1* is able to grow, as the ts *cdc25* mutation is suppressed by *wee1-50*. Multicopy plasmid pDB248' (3) carrying one of the genes indicated was used for transformation of the double mutant, and the growth property of transformants was examined by plating at 26 and 36°C. For the *dis3*$^+$ gene, a plasmid containing the *S. pombe* ADH promoter was constructed and ligated with the *dis3*$^+$ gene. The ADH-*dis3*$^+$ plasmid in addition to the multicopy *dis2*$^+$ and *sds21*$^+$ plasmids reversed the phenotype of the double mutant.

DISCUSSION

In this paper, we report the identification and characterization of the fission yeast *dis3*$^+$ gene product. The cs *dis3-54* mutant appears to enter mitosis but fails to exit from it. The phenotype is reminiscent of that of *dis2-11*, a mutation in the catalytic subunit gene of PP1. Gene cloning by transformation and nucleotide sequence determination demonstrated that the *dis3*$^+$ gene encodes a 970-amino-acid protein. The coding region is interrupted at the 65th position with a 164-bp putative intron. The gene truncation indicates that the NH_2-terminal 74 amino acids are dispensable but that the COOH-terminal 147 amino acids are essential for complementation of cs *dis3-54*. By an immunochemical method, the 110-kDa polypeptide was identified as the *dis3*$^+$ gene product, which existed as salt-extractable oligomers. Immunofluorescence microscopy showed that the dis3$^+$ protein is enriched in the nucleus. Judging from the intensity of the immunoblot band for the adjusted amount of dis3$^+$ protein made in *Escherichia coli*, the amount of dis3$^+$ protein made in *S. pombe* cells is approximately 5×10^3 to 10×10^3 copies per cell, represent-

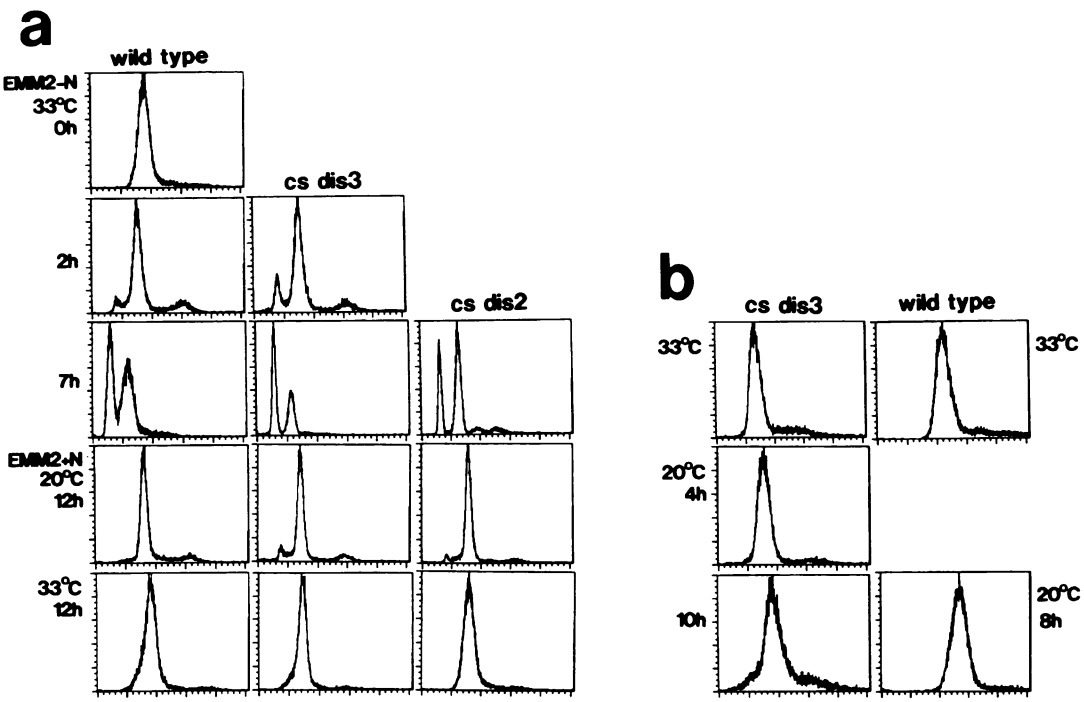

FIG. 9. Transit of the G_1/S phases at a restrictive temperature by *dis3-54* and *dis2-11* mutants. (a) Wild-type, *dis3-54*, and *dis2-11* cells were grown at 33°C in complete EMM2 medium, transferred to EMM2-N medium (lacking a nitrogen source) for 7 h at 33°C, returned to complete EMM2 medium, and incubated for 12 h at 20 or 33°C. Cells were stained with propidium iodide and treated with RNase, and their DNA contents were analyzed with a Becton-Dickinson FACScan. The two peaks present in the culture incubated in EMM2-N for 7 h represent cells with 1c (G_1) and 2c DNA contents. Cells with 1c DNA content disappeared at 20 as well as 33°C 12 h after the readdition of the nitrogen source, indicating that both wild-type and mutant cells can move across the G_1/S phases at the restrictive temperature (20°C). (b) Exponentially growing *dis3-54* and wild-type cultures were transferred to 20°C. Samples were taken after 4 and 10 h and analyzed with the FACScan. All cells analyzed at 10 h contained 2c DNA content.

ing medium abundance. The biochemical activity of the dis3$^+$ protein is not known.

The results of the gene disruption experiments showed that the *dis3*$^+$ gene is essential for cell division and spore germination, consistent with the isolation of a recessive cs mutation. The disruption phenotype, however, was apparently different from that of cs *dis3-54*. Haploid gene disruptant cells carrying a plasmid with the *dis3*$^+$ gene grew but were arrested upon the loss of the plasmid in the rich medium. The characteristic mitosis phenotype of *dis3-54* was not found in these plasmid-deficient cells. Therefore, the *dis3-54* mutant phenotype at the restrictive temperature may be allele specific and may be produced by a partially active mutant protein. The *dis3*$^+$ gene product is perhaps required not only in mitosis but also in other stages of the cell cycle. Although cs *dis3-54* mutant cells can transit the G_1/S boundary at the restrictive temperature, it is still possible that the complete loss of the dis3$^+$ protein may cause arrest at the G_1 phase. This point should be examined by generating more mutations in the *dis3*$^+$ gene.

Several lines of evidence favor the hypothesis that *dis3*$^+$ gene function is involved in the regulation of protein dephosphorylation during mitosis. First, the cell cycle phenotype of the *dis3-54* mutant is similar to that of cs *dis2-11*. The cellular localization of the dis3$^+$ protein is reminiscent of that of the dis2$^+$ protein. Second, *dis2*^{cs} *dis3*^{cs} or *dis2*^{null} *dis3*^{cs} double mutants are not viable, even at the permissive temperature, consistent with the notion that these two genes may have an overlapping function. Third, the *dis3*$^+$ gene under the control of the ADH promoter can reverse the Ts$^+$ phenotype of *cdc25 wee1*, as can the other two PP1 genes. We recently found that the ability of *dis2*$^+$ gene overexpression to reverse the phenotype of *cdc25 wee1* resides in a small domain near the COOH terminus of the dis2$^+$ protein (37a). The full-length PP1 protein is not needed, so the dis3$^+$ protein may directly or indirectly interact with PP1 to affect *cdc25 wee1* regulation of cdc2$^+$ kinase activation. Fourth, the predicted amino acid sequence of dis3$^+$ is similar to that of the budding yeast *SSD1* or *SRK1* gene product (32), which can suppress the lethality attributable to the absence of PPase gene *SIT4* (1). We recently isolated in fission yeast a gene (designated *ppx1*$^+$) which is similar to *SIT4* and will examine the functional relationship between *dis3*$^+$ and *ppx1*$^+$. The phenotypic similarity to *dis2-11*, the synthetic lethality attributable to *dis2-11*, and the high-dosage effect upon *wee1* and/or *cdc25* mutations suggested that the *dis2*$^+$ and *dis3*$^+$ gene products are related. The sequence similarity to budding yeast *SSD1* or *SRK1* indicated the relationship to protein dephosphorylation and phosphorylation.

However, we have also obtained results which do not support the hypothesis. First, the level of PP1 activity monitored as phosphorylase phosphatase is not reduced in *dis3-54* mutant extracts, whereas it is hardly detected in *dis2-11* mutant extracts. However, *dis3-54* may affect a phosphatase having different protein substrates. Second, the *dis3*$^+$ gene product is not complexed with the *dis2*$^+$ gene product. Third, although *dis3*$^+$ and *SRK1* or *SSD1* may be functionally related, the *dis3*$^+$ gene is essential, but the *SRK1* or *SSD1* gene is nonessential. The amino acid sequence identity between *dis3*$^+$ and *SRK1* or *SSD1* is low, so

their relationship remains to be determined. The *dis3* mutation blocks mitosis, but the *SSD1* gene is implicated in the G_1/S transition (32). Therefore, despite the apparent phenotypic similarity of *dis3-54* to *dis2-11*, the defect in *dis3-54* may not be related to protein dephosphorylation. Only future work will elucidate the gene function at the molecular level.

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