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Characterization of *fus1* of *Schizosaccharomyces pombe*: a Developmentally Controlled Function Needed for Conjugation

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In *Schizosaccharomyces pombe*, the *fus1* mutation blocks conjugation at a point after cell contact and agglutination. The cell walls separating the mating partners are not degraded, which prevents cytoplasmic fusion. In order to investigate the molecular mechanism of conjugation, we cloned the *fus1* gene and found that it is capable of encoding a 1,372-amino-acid protein with no significant similarities to other known proteins. Expression of the *fus1* gene is regulated by the developmental state of the cells. Transcription is induced by nitrogen starvation and requires a pheromone signal in both *P* and *M* cell types. Consequently, mutants defective in the pheromone response pathway fail to induce *fus1* expression. The *ste11* gene, which encodes a transcription factor controlling expression of many genes involved in sexual differentiation, is also required for transcription of *fus1*. Furthermore, deletion of two potential Ste11 recognition sites in the *fus1* promoter region abolished transcription, and expression could be restored when we inserted a different Ste11 site from the *mat1-P* promoter. Since this element was inverted relative to the *fus1* element, we conclude that activation of transcription by Ste11 is independent of orientation. Although the *fus1* mutant has a phenotype very similar to that of *Saccharomyces cerevisiae fus1* mutants, the two proteins appear to have different roles in the process of cell fusion. Budding yeast Fus1 is a typical membrane protein and contains an SH3 domain. Fission yeast Fus1 has no features of a membrane protein, yet it appears to localize to the projection tip. A characteristic proline-rich potential SH3 binding site may mediate interaction with other proteins.

Nutritional starvation is the major signal that activates sexual differentiation in the fission yeast *Schizosaccharomyces pombe* (11). As long as the nutritional conditions are favorable, haploid cells will propagate vegetatively, but under conditions of nitrogen starvation, the cells exit from the mitotic cycle and undergo a differentiation process, which requires sexual agglutination, conjugation, nuclear fusion, meiosis, and spore formation to occur in an orderly fashion (see reference 13). The process of conjugation involves the action of diffusible pheromones secreted by *P* and *M* cell types in order to attract each other. When exposed to the opposite pheromone, the cells form projections toward each other (18, 37) and fuse upon cell-cell contact. Attachment at the projection tips between paired cells culminates in localized cell wall degradation and plasma membrane fusion. Nuclear fusion is coordinated with these events, resulting in the formation of a zygote (see reference 53).

In the differentiation process, the pheromones act by binding to specific receptors on the surface of the opposite cell type (29, 60), thereby activating the pheromone response pathway. Transmission of the signal through the pathway involves the actions of the *ras1* function and of three protein kinases encoded by *byr2*, *byr1*, and *spk1* (19, 45, 47, 49, 58, 61, 65) and ultimately activates transcription of pheromone-controlled genes (see reference 48). The transcription factor Ste11 may be a target for this activation, since it is required for expression of many genes involved in sexual differentiation. It acts by binding to a 10-bp T-rich DNA element, the TR box (59). In the promoter of the pheromone-induced gene *mat1-Pm*, deletion of 21 bp containing a TR box abolishes transcription (1). Furthermore, studies of mutations in the TR box suggest that it is essential for pheromone-dependent expression.

The *fus1* function acts during conjugation. Mutant *fus1* cells are blocked at a step following cell contact but prior to cell wall fusion (5, 12, 33). The separating cell walls are not dissolved if both prezygotic partner cells carry this mutation, and only prezygotes will accumulate. The arrest occurs at the G_1 stage of the cell cycle, and the cells can revert to vegetative growth if transferred to fresh medium (15). The *fus1*$^-$ phenotype is easily monitored in prezygotes as the conjugation tubes continue to grow, thereby creating horseshoe-shaped cell pairs. Meiosis is not affected in diploid *fus1* strains.

In the budding yeast *Saccharomyces cerevisiae*, six *FUS* genes have been identified (17, 34, 40, 62). Mutations in these genes cause defects in cell fusion by interfering with the degradation of the cell wall during mating. The *FUS1* gene, which is the best characterized, encodes a 512-amino-acid membrane protein (63). Transcription of *FUS1* occurs only in haploid **a** and α cells and is strongly induced by exposure to pheromone (40, 62). Consistent with this is the observation that *FUS1* transcription depends on the components in the pheromone response pathway (40): *STE4*, which encodes the β subunit of a G protein; *STE7*, *STE11*, and *FUS3*, which all encode protein kinases; and *STE5*, which has an unknown function (reviewed in reference 35). Transcription of *FUS1* also requires *STE12*, a transcription factor that binds to the pheromone response element (PRE) (10). In the *FUS1* promoter, four PREs have been identified and deletion of all four PREs prevents *FUS1* expression (22).

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

Strain	Genotype	Source or reference
EG 282	h^{90}	50
EG 325	h^{90} *ura4-D18*	49
EG 382	h^{90} *fus1-B20 leu1 ade6-M210 ura4-D18*	This study[a]
EG 385	h^{90}*-B102 fus1-B20 ade6-M216 ura4-D18*	This study
EG 439	h^{90} *fus1*::*ura4*$^+$ *ura4-D18*	This study
EG 459	h^{90}/h^{90} *fus1*::*ura4*$^+$*/fus1*$^+$ *ura4-D18/ura4-D18*	This study
EG 494	h^{90} *ste11 leu1 ade6-M210*	59
EG 495	h^{90} *leu1 byr1*::*LEU2*	30
EG 544	h^- Δ*mat2,3*::*LEU2*	49
EG 545	h^+ Δ*mat2,3*::*LEU2*	16
EG 559	h^{90} *ste8*::*ura4*$^+$ *ura4-D18*	58
EG 679	h^{90} *fus1*::*ura4*$^+$ *ade6-M216 ura4-D18*	This study
EG 680	h^{90} *fus1-B20 leu1*	14
EG 712	h^{90} *fus1-B20*	This study
EG 713	h^+ Δ*mat2,3*::*LEU2 fus1-B20*	This study
EG 754	h^- Δ*mat2,3*::*LEU2 fus1-B20*	This study
EG 794	h^+ Δ*mat2,3*::*LEU2 cyr1*::*ura4*$^+$ *ura4-D18*	9
EG 796	h^- Δ*mat2,3*::*LEU2 cyr1*::*ura4*$^+$ *ura4-D18 sxa2*	This study

[a] The *fus1-B20* allele was described by Bresch et al. (5).

As part of a study aimed at understanding the molecular mechanism of conjugation in *S. pombe*, we further characterized the *fus1* mutant and cloned the gene by complementation. Analysis of the regulation of the *fus1* gene shows that pheromones cause a strong induction of the transcript. Consistently, we observed that transcription requires an intact pheromone response pathway. The *fus1* upstream region contains two TR boxes. Our results suggest that these sequences are necessary for *ste11*-mediated transcription of *fus1*.

MATERIALS AND METHODS

Strains, media, and genetic methods. The *S. pombe* strains used are listed in Table 1. Standard classical and molecular genetic techniques for *S. pombe* were used as described previously (20, 44). Protoplast fusions were done essentially as described previously (46). The media PM and PM−N used for growing *S. pombe* cells were prepared as described in reference 3. Minimal sporulation agar (MSA) was prepared according to reference 16. *Escherichia coli* DH5 (23) was used for routine growth and maintenance of plasmids. Recovery of plasmids from *S. pombe* into *E. coli* JA226 has been described previously (2). Standard procedures for manipulating of DNA were used according to reference 57. PCR amplification was carried out in 50-μl reaction mixtures (31).

Quantitative mating assay. The cells were grown in PM to a density of 5×10^6 cells per ml, mixed in a 1/1 ratio. (Homothallic strains were grown to a density of 5×10^6 cells per ml.) Five microliters was spotted on an MSA plate, and the cells were incubated for 48 h. Mating frequencies were calculated by inspection under the microscope. More than 500 cells were counted in each cross. The efficiency of mating was calculated as (2 × number of asci and zygotes formed)/[(total number of cells) + (2 × number of asci and zygotes)].

Cloning of *fus1*. *S. pombe* EG 382 (h^{90} *fus1-B20 leu1 ade6-M210 ura4*) was transformed with a *Bcl*I genomic DNA library in the vector pON163 (66). Ura$^+$ transformants were selected and then replica plated to a lawn of strain EG 385 (h^{90}*-B102 fus1-B20 ade6-M216 ura4*). The plates were incubated for 24 h at 30°C and then replica plated to MSA. The *B102* mutation in the *mat1-Pm* gene increases the fraction of zygotes that will resume diploid mitosis (12). The mutations *ade6-M216* and *ade6-M210* complement each other intragenically, so that only diploid cells resulting from conjugation can grow. Cloning of the *ade6* gene was avoided because the gene bank was constructed from an *ade6-M210* mutant (66). The plates were washed and the vegetative cells were killed with 30% ethanol (36). The ascospores were then spread on minimal plates. From an iodine-positive colony containing only zygotic asci and therefore expected to be *fus1*$^+$, a recombinant plasmid was isolated and designated pDW220.

Sequence analysis. Sequencing was carried out by the dideoxy chain termination method with ^{35}S-labelled nucleotides and a Sequenase kit (U.S. Biochemical Corp.). Double-stranded plasmid DNA inserted in pGEM plasmids was used as a template. Two sets of overlapping unidirectional deletions, generally 100 to 200 bp apart, were generated by treating pDW235, pDW234 (see Fig. 3A), and pDW372 (insert is identical to pDW236) with exonuclease III and nuclease S1 as described previously (24). The primers used were the standard sequencing primers homologous to the SP6 and T7 promoter sequences in the pGEM3 plasmid (Promega). Specific primers were also made. Both strands of the sequence presented in Fig. 4 were determined entirely from overlapping clones or specific primers.

Northern (RNA) analysis. Cells were grown at 30°C in PM to a density of 5×10^6 cells per ml, harvested, and resuspended in PM and PM−N at the same density. After 5 h of incubation at 30°C, RNA was isolated as described previously (50). The effect of mating pheromones on *fus1* expression was monitored by adding 300 U of synthetic M factor (64) or P factor (27) per ml to mitotically growing *cyr1*$^-$ cells for 5 h (10^7 cells per ml).

Northern blot analysis was essentially performed as described in reference 30. Hybridization to single-stranded RNA probes and preparation of *cdc2*-specific probes were performed as described previously (49). Single-stranded *fus1*-specific RNA probes were transcribed from the pGEM3 vector containing a *Hin*dIII-*Xho*I *fus1* fragment (pDW375 [Fig. 3A]).

Deletion analysis of the *fus1* promoter region. Deletions were constructed as shown in Fig. 8. All plasmids are pDW227 derivatives (66), and all contain a 1,700-bp truncated *fus1* gene under control of the altered promoter. In order to integrate the plasmids at the *fus1* locus, the plasmids were linearized with *Cla*I (Fig. 8A). The integration was done in EG 325 (h^{90} *ura4D18*) and confirmed by PCR with a primer recognizing the SP6 promoter in the plasmid and JPP2 (GGGGATCCATTGCAGTTGGTAAAGA), which is specific for *fus1* downstream of the integration point. pJP20 contains an unmodified *Hin*dIII-*Eco*RI fragment from pDW220 cloned into pDW227. The plasmid pJP31 is a pDW235 derivative (Fig. 3A) generated by exonuclease treatment. In pJP36, the truncated *fus1* gene was cloned as an *Eco*RI-*Eco*RI fragment by PCR with the use of pDW220 as template and two *fus1*-specific primers, JPP14 (N terminal [5′-GCG**GAATTC**CGATGACGGCTAGTTTT], which contains an *Eco*RI site, and JPP2 (C terminal [5′-GGGGATCCATTGCAGTTGGTAAAGG], which is located downstream of the *Eco*RI site in the *fus1* open reading frame (ORF). Deletion of the two TR boxes TR1 (−238 relative to the translation start) and TR2 (−156 relative to the translation start) in pJP34 and pJP41 was done by PCR. The sequences upstream of TR1 and TR2, respectively, were amplified with the same template (pJP20) and the same upstream primer (the SP6 primer, which recognizes sequences in the vector) and then two different *fus1* primers upstream of TR1 (JPP6) or TR2 (JPP15), both reading away from the ORF. The sequences downstream of TR1 and TR2, respectively, were also amplified with the same template (pDW220) and the same downstream primer (JPP2 described above) and two *fus1*-specific primers downstream of TR1 (JPP7) or downstream of TR2 (JPP11). Primers specific for pJP34 (deletion of TR1) contain a *Bam*HI site: JPP6 (5′-CGG**GGATCC**ATACTAACTGTGTTCG) and JPP7 (5′-CGC**GGATCC**TTTAACGGGTACAATA). Primers specific for pJP41 (deletion of TR2) contain a *Kpn*I site: JPP15 (5′-CGC**GGTACC**GGTTTAACCGACGTAA) and JPP11 (5′-CGC**GGTACC**AGTTATGTTCTAAATA). The PCR-amplified fragments were then inserted in pDW227 digested with *Hin*dIII and *Eco*RI. For construction of pJP49, the sequence upstream of TR2 was cloned by PCR with the primers SP6 JPP15 and with pJP34 as the template. This amplified fragment, in which TR1 had already been deleted, was cloned into pJP41 digested with *Hin*dIII and *Kpn*I. In pJP53, the synthetic TR box was inserted by blunt end ligation. pJP49 was digested with *Bam*HI and filled in with the Klenow fragment, and the primers ONP31 (5′-CTAA**TGCTTTGTTC**CCTCTTT) and ONP32 (5′-AAAGAGG**GAACAAAGCA**TTAG) were annealed and cloned into it. Restriction sites and TR boxes contained in particular primers are indicated by boldface type.

Construction of HA-*fus1* tags and Western blot (immunoblot) analysis. The hemagglutinin (HA)-*fus1* fusion protein expression vector (pJP54) was constructed as follows. The autonomously replicating plasmid pART1 (41), containing *S. cerevisiae LEU2* as a selective marker and the constitutive *adh1* promoter, was used as a vector. A *Sac*I-*Sac*I fragment containing the HA epitope (42) was cloned into a different polylinker in order to facilitate subcloning as a *Pst*I-*Eco*RI fragment (pIL117). Fusion of the C terminus of Fus1 with the HA epitope was done by PCR with pDW220 as the template and two *fus1*-specific primers, JPP14, containing an *Eco*RI site, which was used for fusion in frame with the HA epitope, and JPP2, which is located downstream of the *Eco*RI site in the *fus1* ORF. This *Eco*RI-*Eco*RI fragment was subcloned into pIL117, resulting in pJP50. Finally, the HA-*fus1* fusion from pJP50 (a *Pst*I-*Xho*I fragment) and the C terminus of Fus1 (a *Xho*I-*Bam*HI fragment from pDW375 [Fig. 3B]) was cloned into pART1 (=pJP54).

The triple HA-Fus1 fusion protein was constructed as follows. The *fus1* promoter was PCR amplified with two *fus1*-specific primers, ONP95 (5′-GG**GATCC**AAAACACATGCCATCGG) and ONP102 (5′-GCGG**GTACCGTCGA C**TAACAAGCAATAGCT), and with pDW220 as the template. This fragment was cloned into a polylinker in order to facilitate subcloning of a *Pst*I-*Sal*I fragment into *Pst*I-*Xho*I-digested pSLF173 (kindly provided by Susan Forsburg), which results in fusion of the *fus1* promoter to the triple HA tag (pJP59). The *fus1* ORF was subcloned from pJP50 as a *Sma*I-*Eco*RI fragment and fused in frame after the triple HA tag in pJP59 (=pJP60). This fusion protein contains the same part of the Fus1 ORF as the one encoded by pJP54. pJP60 was linearized with *Cla*I and integrated in EG 325 (h^{90} *ura4D18*). The integration was confirmed by PCR with the two *fus1*-specific primers ONP95, recognizing *fus1*

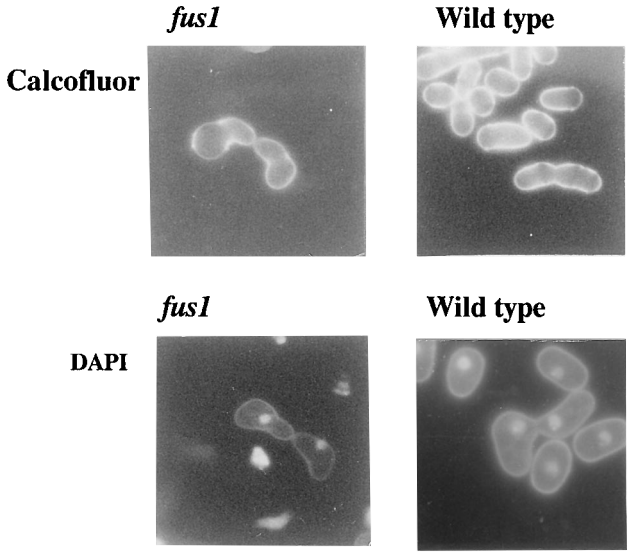


FIG. 1. Calcofluor staining of the cell walls demonstrates that they are undegraded in the *fus1* mutant. Staining of the nuclei with DAPI reveals that they are located at medial positions in the prezygotic cells, in contrast to wild-type zygotes, where they move towards the projection tips.

(see above), and JPP2 (5′-GGGGATCCATTGCAGTTGGTAAAGA), which is specific for the *fus1* sequence downstream of the integration point. Before electrophoresis, the PCR product was digested with enzymes specific for the triple HA tag DNA.

Protein extracts were prepared from exponentially growing cultures according to reference 51. Western blot analysis was performed according to reference 42, except the blot was developed by using the Amersham ECL Western blotting detection reagents.

Cytological techniques. For immunofluorescence microscopy, cells were grown in PM to a density of 5×10^6 cells per ml. After 10 h of nitrogen starvation in PM−N, fixation with aldehydes and the antibody staining were performed according to reference 21. The HA-specific 12CA5 antibody (42) was used as the primary antibody. The secondary antibody used was affinity-purified goat anti-mouse immunoglobulin G, which was whole-molecule fluorescein isothiocyanate conjugated (E. Y. Laboratory [kindly provided by Iain Hagan]).

Staining of the prezygotes with calcofluor and DAPI (4′,6′-diamidino-2-phenylindole) was performed with an exponentially growing culture at approximately 5×10^6 cells per ml, which was starved in PM−N for 10 h. The cells were washed with H_2O and resuspended in H_2O containing the fluorescent agent. The DNA-specific dye DAPI was added at 1 μg/ml, and the cell wall-specific dye calcofluor was added at 10 mg/ml.

Nucleotide sequence accession number. The GenBank accession number for the *fus1* sequence is L37838.

RESULTS

***fus1* affects only conjugation.** The *fus1* mutant fails to dissolve or reorganize the cell walls between the mating partners (see Fig. 2C). This partition prevents mixing of cytoplasmic organelles and nuclear fusion. Calcofluor staining of the cell walls in *fus1* prezygotes demonstrates that the walls are intact between the mating pairs (Fig. 1). This is in agreement with the fact that *fus1* prezygotes can revert to vegetative growth when transferred to fresh medium (15). DAPI staining of the nuclei reveals that they are located on opposite sides of the partition, usually not close to the tips (Fig. 1), in contrast to wild-type cells, where the nuclei move toward the projection tips (6).

In a quantitative mating assay, the effect of carrying the *fus1* mutation in one or both mating types was monitored. Wild-type and mutant *fus1* heterothallic strains of both mating types were mixed in a 1:1 ratio and then spread on MSA plates. Mating frequencies of the h^+ strains EG 545 and EG 713 with the h^- strains EG 544 and EG 754 were calculated by inspection under a microscope (Table 2).

TABLE 2. Mating efficiencies of mutants defective in *fus1*

h^+ strain	Efficiency of mating (%) with h^- strain[a]:	
	EG 544 (*fus*$^+$)	EG 754 (*fus1*$^-$)
EG 545 (*fus*$^+$)	40.5 ± 5.8	5.8 ± 0.2
EG 713 (*fus1*$^-$)	7.1 ± 0.2	0[b]

[a] Values represent means of at least three separate trials ± standard deviations.

[b] The *fus1-B20* mutation is slightly leaky. When it was present in a homothallic strain, we found a mating efficiency of 1.9% ± 0.3%. This higher value probably reflects the fact that mating-type switching leads to pairs of sister cells having opposite mating types (43) and hence improves pairing.

The experiment shows that mating between two *fus1* mutants is completely blocked; only abnormal prezygotes are present. Mating of a wild-type cell with a *fus1*$^-$ partner produced normal zygotes, independent of which mating type was *fus1*$^-$. This indicates that provision of *fus1* activity from one side is sufficient for the breakdown of both separating cell walls, although the mating frequency is decreased compared with mating between two wild-type strains. This experiment suggests that the *fus1* gene can be expressed in both mating types. We further examined the *fus1* mutant for defects in other processes during sexual differentiation. The cells responded normally to pheromone by elongation and shmoo formation, they agglutinated normally, they produced normal amounts of pheromone, and, if they were made diploid by protoplast fusions, they sporulated as wild-type cells, suggesting that the mutation does not affect meiosis (data not shown). All of this is evidence that *fus1* has a function only in cell fusion.

Cloning of *fus1* by rescue of the mutant phenotype. We first attempted to clone the *fus1* gene simply by restoration of sporulation to an h^{90} *fus1-B20* mutant strain. This procedure, however, gave rise to a large number of false-positive sporulating diploid colonies (data not shown). We therefore designed a strategy that directly took advantage of the mating defect of the *fus1* mutant. The experimental approach was based on intragenic complementation of two *ade6* mutants and on the fact that provision of wild-type Fus1 activity from one side is sufficient for the breakdown of both separating cell walls (described above). The *fus1-B20* mutant was transformed with a fission yeast genomic library with *ura4* as a selective marker. Ura$^+$ transformants were pregrown on minimal plates and mated to lawns of *fus1*$^-$ cells and then replica plated to medium on which only the fraction of the transformants that had formed diploids could grow. In order to eliminate false positives, due to the slight leakiness of *fus1-B20*, free ascospores of the selected diploids were plated and the descending haploid colonies were screened for the Fus$^+$ phenotype. Several Ura$^+$ and Fus$^+$ clones were obtained. Vegetative progeny from these showed simultaneous loss of the Ura$^+$ and Fus$^+$ phenotypes when grown in nonselective medium, indicating that complementation of the two markers was caused by a single autonomously replicating plasmid. Plasmid DNA isolated from one of these transformants was recovered in *E. coli*. Upon retransformation, this plasmid (pDW220) was able to complement the *fus1* mutation, suggesting that it contained the *fus1* gene. Figure 2D shows the complementation observed with pDW220.

To define more precisely the boundaries of the complementing region of pDW220, different fragments of the nuclear DNA insert were cloned into the shuttle vector pDW232 (66), and the constructs were tested for complementation of the mating deficiency of the *fus1-B20* allele (Fig. 3B). The region between the left *Hin*dIII site and the right *Xho*I site present in pDW375

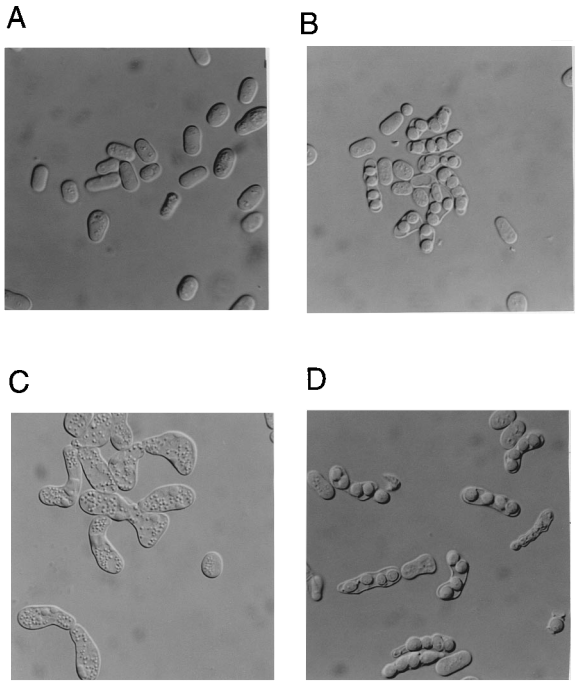


FIG. 2. Sexual differentiation in *S. pombe*. The cells were starved of nitrogen for 48 h on MSA. (A) Heterothallic h^+ strain (EG 545); (B) homothallic h^{90} strain (EG 282); (C) h^{90} *fus1-B20* mutant strain (EG 382); (D) h^{90} *fus1-B20* mutant strain (EG 382) complemented by *fus*$^+$ plasmid (pDW220). Only the homothallic strain and the mutant strain transformed with pDW220 are sporulating.

proved to be sufficient for restoring the Fus$^+$ function to *fus1* mutant cells (Fig. 3B). Southern hybridization of total DNA restricted with *Eco*RV or *Xho*I showed that the cloned DNA insert was derived from a unique sequence in the *S. pombe* genome (data not shown).

Disruption of the *fus1* gene and sequence analysis. To confirm genetically that pDW220 actually contained the *fus1* gene and not an extragenic suppressor, the cloned segment was used to direct a disrupting selectable marker into the *fus1* locus at the right arm of chromosome I (14). A disrupted *fus1*::*ura4* allele was constructed by inserting a 1.7-kb *Cla*I fragment containing the *S. pombe ura4* gene into the *Cla*I site of pDW234 (Fig. 3C). The resulting plasmid was digested with *Xho*I, and the fragment containing *ura4* was used for transformation of a homothallic diploid strain carrying the *ura4* mutation (EG 325-2n). Several transformants were obtained, and integration of this construct at the chromosomal *fus1* locus was confirmed by Southern analysis (*Eco*RV-digested chromosomal DNA hybridized with a probe transcribed from pDW234 [data not shown]). Tetrads of the hybrid diploids were dissected. Each ascus contained four viable spores, indicating that the *fus1* gene is not essential for mitotic growth. The tetrad data showed regular 2:2 segregation for both the Fus$^+$/Fus$^-$ and Ura$^-$/Ura$^+$ phenotypes. As expected, close linkage between *ura4* and *fus1* was demonstrated (data not shown). The phenotype of transformants carrying the disrupted segment was found to be Fus$^-$. This *fus1* allele seems to be tight compared with *fus1-B20*, which is slightly leaky (as described above and shown in Fig. 8). Additionally, we made the disruption in a haploid strain and protoplast fused this strain (EG 679) with the strain carrying the *fus1-B20* allele (EG 680). Sporulation of the resulting diploid strain gave rise to only Fus$^-$ progeny, which confirms that the cloned fragment actually is the *fus1*$^+$ gene.

The DNA sequence of the 5-kb *fus1*-complementing *Hin*dIII-*Xho*I fragment from pDW375 was determined. Analysis of the sequence revealed the presence of a 1,372-amino-acid uninterrupted ORF (Fig. 4). A database search revealed no significant similarities to other known proteins, including *S. cerevisiae FUS1* (62). We found an mRNA species corresponding to the *fus1* coding strand (described below). The transcription start was determined by primer extension to be located at position −32 relative to the translation start (Fig. 4 [data not shown]). This means that the *fus1* mRNA starts with AUG. Observation has shown that translation initiates at the first AUG codon present in 90% of eukaryotic mRNAs (32). However, it seems unlikely that translation can be initiated at the first codon in

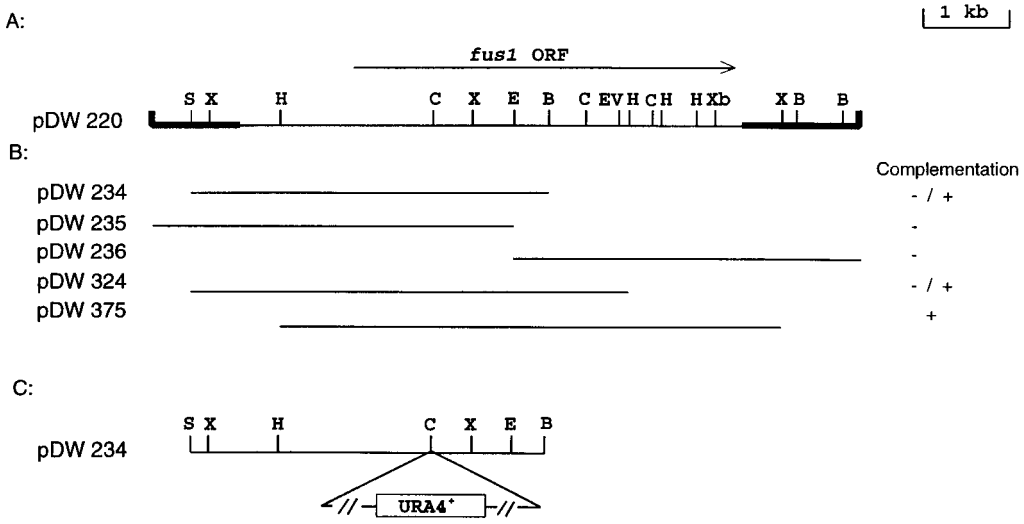


FIG. 3. (A) Restriction map of the *fus1* gene (pDW220). B, *Bgl*II; C, *Cla*I, E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; S, *Sal*I; X, *Xho*I; Xb, *Xba*I. The extent and direction of the *fus1* ORF are shown by an arrow. The thin line indicates the portion which has been sequenced in both directions. (B) Complementation analysis, in which pDW375 complements the *fus1* strain. (C) Construct used for one-step disruption.

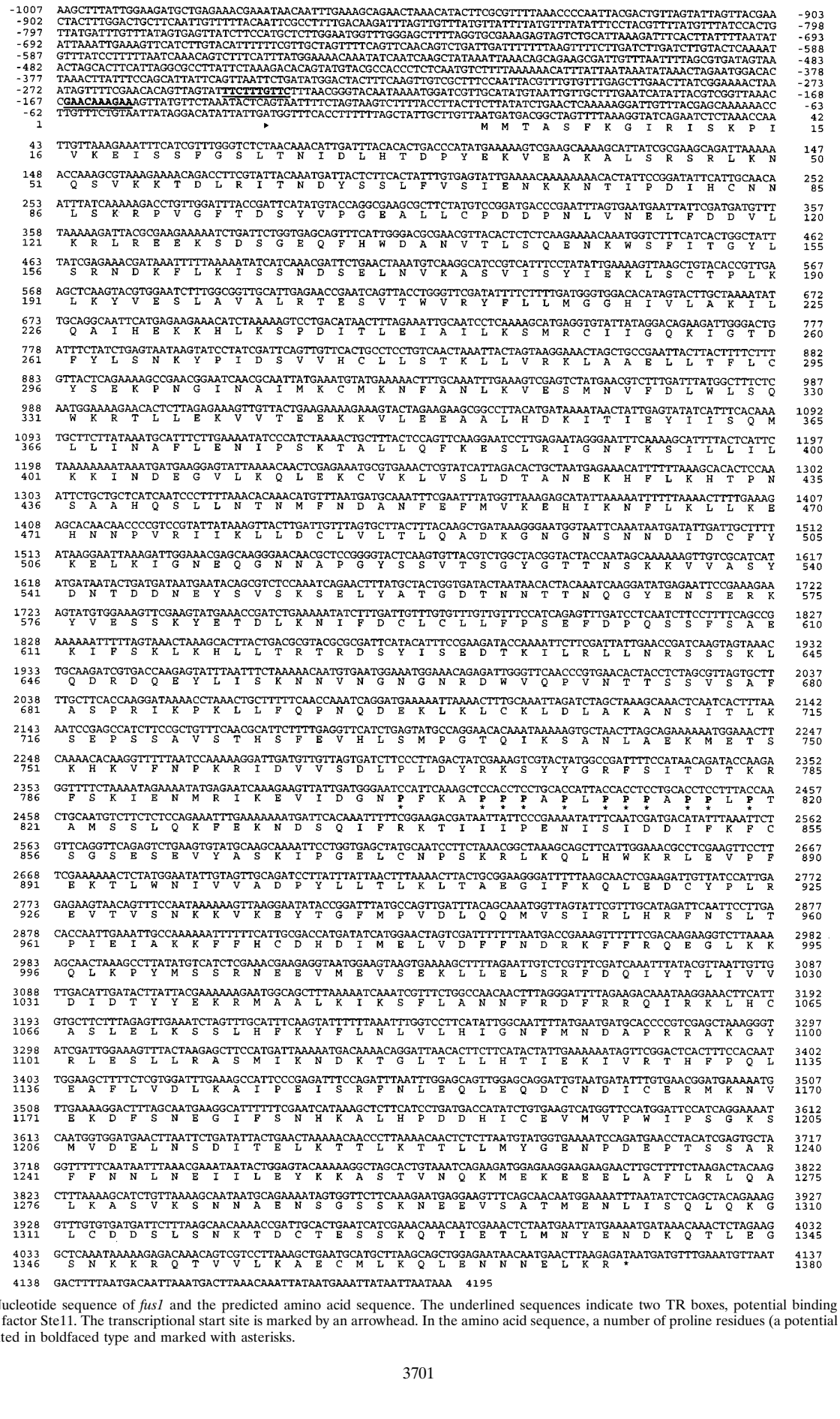


FIG. 4. Nucleotide sequence of *fus1* and the predicted amino acid sequence. The underlined sequences indicate two TR boxes, potential binding sites for the transcription factor Ste11. The transcriptional start site is marked by an arrowhead. In the amino acid sequence, a number of proline residues (a potential SH3 binding site) are printed in boldfaced type and marked with asterisks.

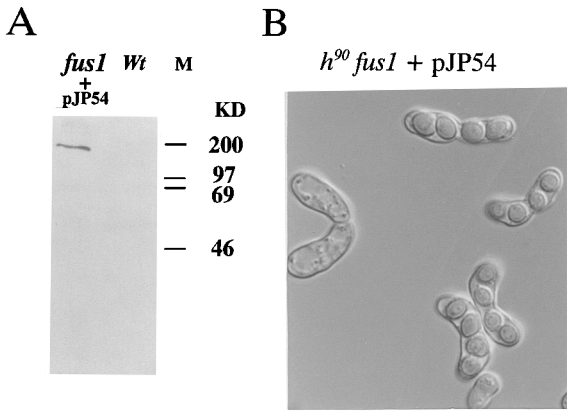


FIG. 5. (A) Identification of the HA-*fus1* fusion protein by Western blot analysis. Primary antibody against HA was used for detection, and the only band that appeared had a size which corresponds to that of the *fus1* ORF (about 160 kDa). The plasmid containing the fusion protein complements the *fus1*$^-$ strain EG 382. M, molecular mass standards (kilodaltons). (B) Complementation of the *fus1*$^-$ strain (EG 382) by HA-tagged Fus1.

this messenger, and furthermore the product would be out of frame with the ORF. Therefore, translation is likely to initiate at the indicated second AUG codon, but it could also be initiated at the AUG codon just downstream of it. In that case, there would be an adenine residue at position −3, which has been shown to be important for efficient translation in *S. cerevisiae* (8).

Induction of *fus1* requires a pheromone signal. Using strand-specific RNA probes, we performed Northern analysis to determine whether the level of *fus1* mRNA was induced during conjugation. Figure 7 shows that four *fus1*-specific bands can be detected (h^{90} − *N*). The largest band has a size of approximately 4 kb, which corresponds to the size of the ORF. This band was highly induced in lanes with RNA prepared from starved homothallic cells, and we propose that it is the primary *fus1* transcript. Two smaller bands presumably represented decay or processing products of the *fus1* transcript, which were concentrated at the leading edge of the massive 18S and 25S rRNA bands (25). Their appearance seemed to correlate with that of the *fus1* band. Another band, which showed no induction by starvation, was present below the 25S rRNA in all lanes. Presumably this was due to cross-hybridization to a transcript of an unknown gene, which appears to be more abundant in heterothallic strains. We have fused the HA epitope to the N terminus of the *fus1* reading frame (see Materials and Methods). When expressed from the constitutive *adh* promoter, this construct fully complements *fus1*, and a Western blot of a strain transformed with it revealed only one band with a size predicted by the ORF (Fig. 5). Thus, the 4-kb band observed in Northern blots appears to give rise to the Fus1 protein.

The *fus1*$^+$-mediated degradation of cell walls is expected to function only during the process of mating. The activation of this function could be due to either transcriptional or translational induction or might be caused by posttranslational modifications of the gene product. We found that the *fus1* gene is only very weakly expressed in mitotic cells (Fig. 6A, h^{90} + N). In a homothallic strain, activation of mating by nitrogen starvation caused a strong induction of *fus1* transcription (Fig. 6A, h^{90} − N). Heterothallic strains failed to activate the *fus1* gene upon nitrogen starvation (Fig. 6A, h^- − N and h^+ − N), which indicates that transcription of the *fus1* gene may depend on a pheromone signal. To test this directly, we added purified pheromones to heterothallic *cyr1* strains (Fig. 6B). Such strains can respond to pheromones in rich medium (9, 38, 68). The h^- strain also carried the *sxa2* mutation in order to prevent degradation of P factor (26). Addition of both P factor to the h^- strain and M factor to the h^+ strain caused a strong induction

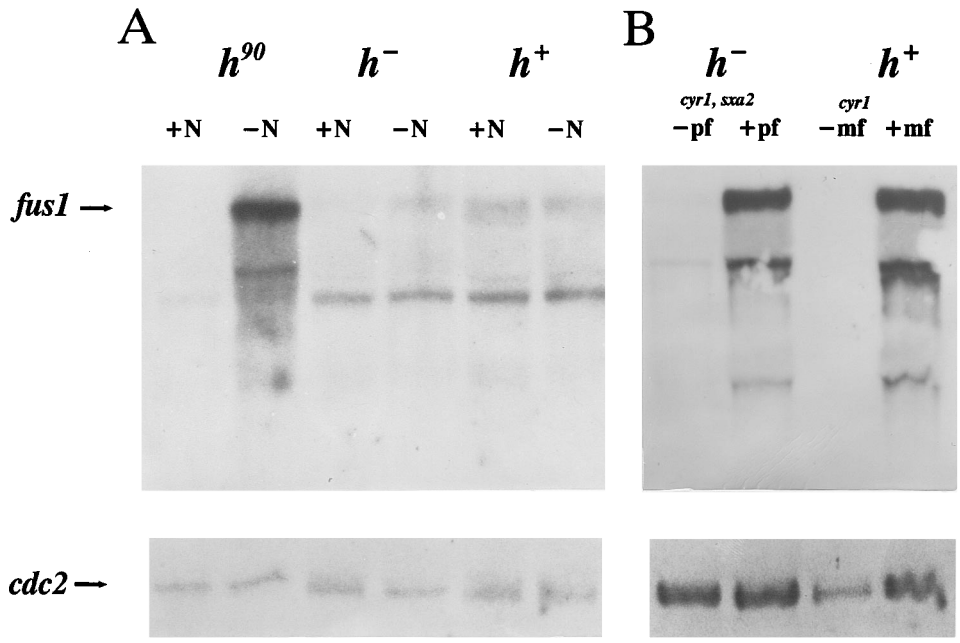


FIG. 6. Transcriptional analysis of the *fus1* gene. (A) A homothallic h^{90} strain (EG 282) and heterothallic h^+ (EG 545) or h^- (EG 544) strains were grown in liquid minimal medium with or without nitrogen present. The 4-kb *fus1* transcript was only expressed in nitrogen-starved homothallic strains. (B) Addition of either P or M factor to heterothallic *cyr1* strains (EG 794 and EG 796) caused a strong induction of the *fus1* transcript. The same membranes were also hybridized to a *cdc2*-specific probe (bottom panels).

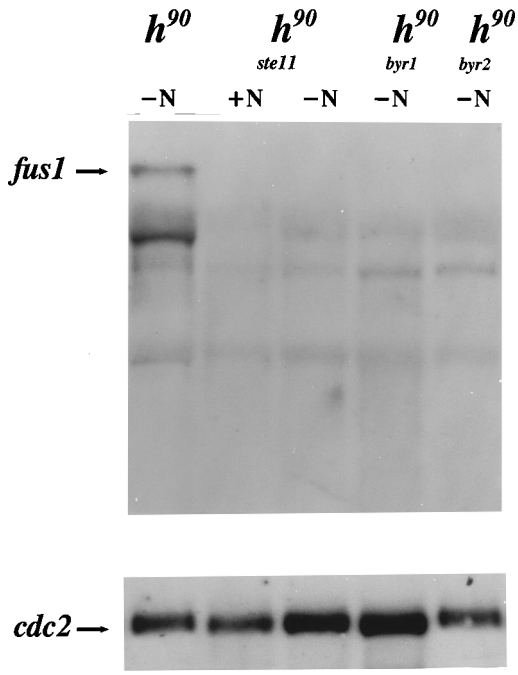


FIG. 7. Effects of interruption of the pheromone response pathway and deletion of the Ste11 transcription factor on *fus1* transcription (top panel). The strains tested were EG 282 (h^{90}), EG 494 (h^{90} *ste11*), EG 495 (h^{90} *byr1*), and EG 559 (h^{90} *byr2*). The membrane was also hybridized to a *cdc2*-specific probe (bottom panel).

of the *fus1* transcript. Hence, we conclude that the *fus1* gene is pheromone controlled. Furthermore, the *fus1* gene can be expressed in both mating types.

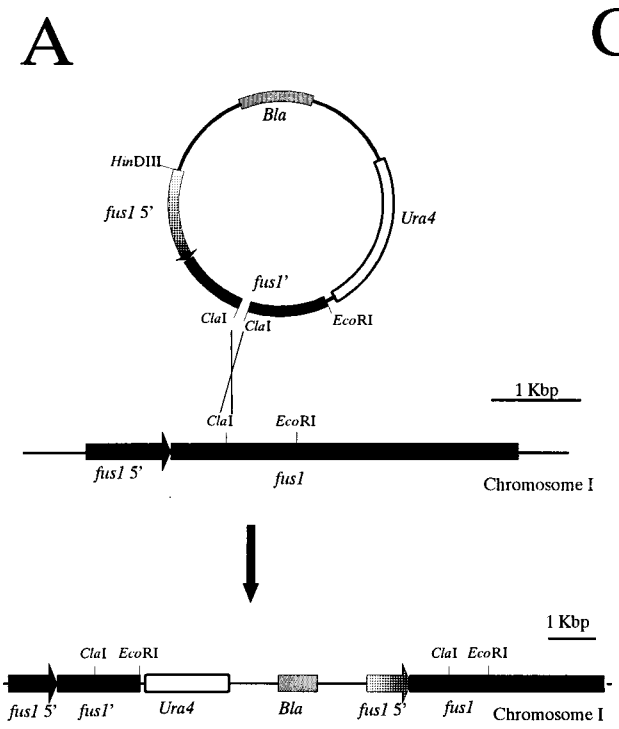
Transcription of the *fus1* gene depends on an intact pheromone response pathway. Expression of pheromone-controlled genes, such as *mat1-Pm*, is abolished in cells defective in components of the pheromone response pathway (e.g., *map3*, which encodes the M factor receptor; *gpa1*, which encodes the α subunit of the G protein; *ras1*, which is a GTP-binding protein; or *byr2*, *byr1*, and *spk1*, which all encode protein kinases). Furthermore, transcription is abolished in a *ste11* strain, which is defective in a transcription factor (1, 19, 45, 49, 58–61, 65). It was therefore of interest to investigate whether defects in these genes affected *fus1* transcription. By Northern analysis of nitrogen-starved cells, we found that mutations in *byr2*, *byr1*, or *ste11* abolished transcription of the *fus1* gene (Fig. 7). The effect of *map3*, *gpa1*, and *ras1* mutations on *fus1* transcription was also investigated, and all of these mutants failed to induce the transcript upon nitrogen starvation (data not shown). We therefore conclude that expression of *fus1* depends on an intact pheromone response pathway.

Function of the TR box is orientation independent. In the *fus1* 5′-upstream region, we noticed a potential positive regulator sequence (TR1) at position −238 relative to the translation start (Fig. 4). This element is a TR box (TTCTTTGTTY), which is the recognition site of the transcription factor Ste11, and it is found in the promoter regions of many genes induced under sexual differentiation: *mei2*, *matP*, *matM*, and *ste11* itself (59). In the *mei2* promoter and in the *mat1-Pm* promoter, the TR boxes were shown to play a critical role in controlling expression of these genes (1, 59). Accordingly, we expected the TR box in the *fus1* 5′-upstream region to be involved in control of expression and therefore deleted it. In order to measure the effect of this deletion or others in the promoter region, we used an in vivo assay for *fus1* transcription (Fig. 8). The mutations were constructed in plasmids intended to integrate at the *fus1* locus at the right arm of chromosome I (14). They contained a truncated Fus1 gene under control of the mutated promoter and *ura4* as a selective marker. As a result of the integration event, the altered promoter will control the wild-type *fus1* reading frame, and a mating defect will result if transcription is substantially reduced. First, we showed that the unmodified promoter fragment (pJP20) was sufficient to give rise to wild-type Fus^+ activity (Fig. 8B and C1). As a control (Fig. 8C2) we observed that a promoterless *fus1* gene (pJP36) could not give rise to Fus^+ activity. Figure 8B shows a strong decrease in mating efficiency corresponding to that of the original *fus1-B20* mutant. Deletions in the promoter to a position just upstream of the TR box (pJP31) had no effect on the mating efficiency, and, surprisingly, a deletion of the TR box itself (pJP41) had no effect either. We therefore looked for additional TR elements, and at position −157 relative to the translation start, we found another TR box (TR2) situated in the opposite orientation. Deletion of this one alone (pJP41) had no effect either, but when both TR boxes were removed simultaneously (pJP49), mating was strongly reduced (Fig. 8B and C3). To confirm that the reduction in mating was actually caused by deletion of TR1 and TR2, we tried to restore mating by insertion of a different TR box (pJP53). This TR box was identical to one from the *mat1-P* promoter. Figures 8B and C4 show that insertion of this TR box indeed restored mating to the same level as that seen in pJP41. Furthermore, the synthetic TR box was inserted in the opposite orientation compared with the wild-type TR box at this position. Hence, we conclude that at least one of the two TR boxes in the *fus1* promoter is required for expression.

The Fus1 protein associates with the projection tips. We wanted to determine the localization of the Fus1 protein within conjugating cells by indirect immunofluorescence microscopy with primary antibodies against the HA tag (67). First, we looked at nitrogen-starved homothallic cells expressing the HA-*fus1* fusion protein from the strong constitutive *adh* promoter on a plasmid. As mentioned above, these conditions fully restored conjugation in *fus1* mutant cells (Fig. 5B). The signal appeared to be mainly cytoplasmic, and we were unable to detect any asymmetrical distribution of the protein within the cells (data not shown). However, since these results were obtained with cells overexpressing the protein, we could not rule out the possibility that this obscured a specific localization of the protein. We therefore proceeded to express the fusion protein from the *fus1* promoter in single copy in the genome. In order to increase the sensitivity of detection, we used the triple HA tag (17a). To our surprise, the *fus1* mutant was no longer complemented under these conditions (data not shown). However, the protein now showed a specific association with the prezygotic projection tips in conjugating cells (Fig. 9). Presumably, the activity of the protein is reduced by the presence of the tag, and overexpression can compensate for this. The fact that the fusion protein localizes to the projection tip suggests that a specific mechanism that directs Fus1 to the point of cell fusion exists and that although the tagged protein can no longer mediate cell fusion, it has retained this ability.

DISCUSSION

At the biochemical level, very little is understood about the actual steps of zygote formation in *S. pombe*. Once in firm contact, the cell walls and plasma membranes separating the two G_1-arrested cells must be removed to permit cell fusion.


A
Bla
HinDIII
fus1 5'
Ura4
fus1'
ClaI
ClaI
EcoRI
1 Kbp
ClaI
EcoRI
fus1 5'
fus1
Chromosome I
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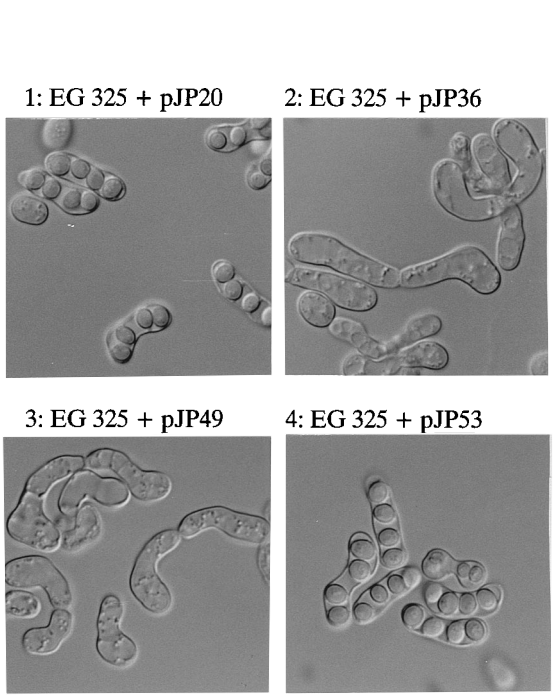
fus1 5'
fus1
Chromosome I


C
1: EG 325 + pJP20
2: EG 325 + pJP36
3: EG 325 + pJP49
4: EG 325 + pJP53

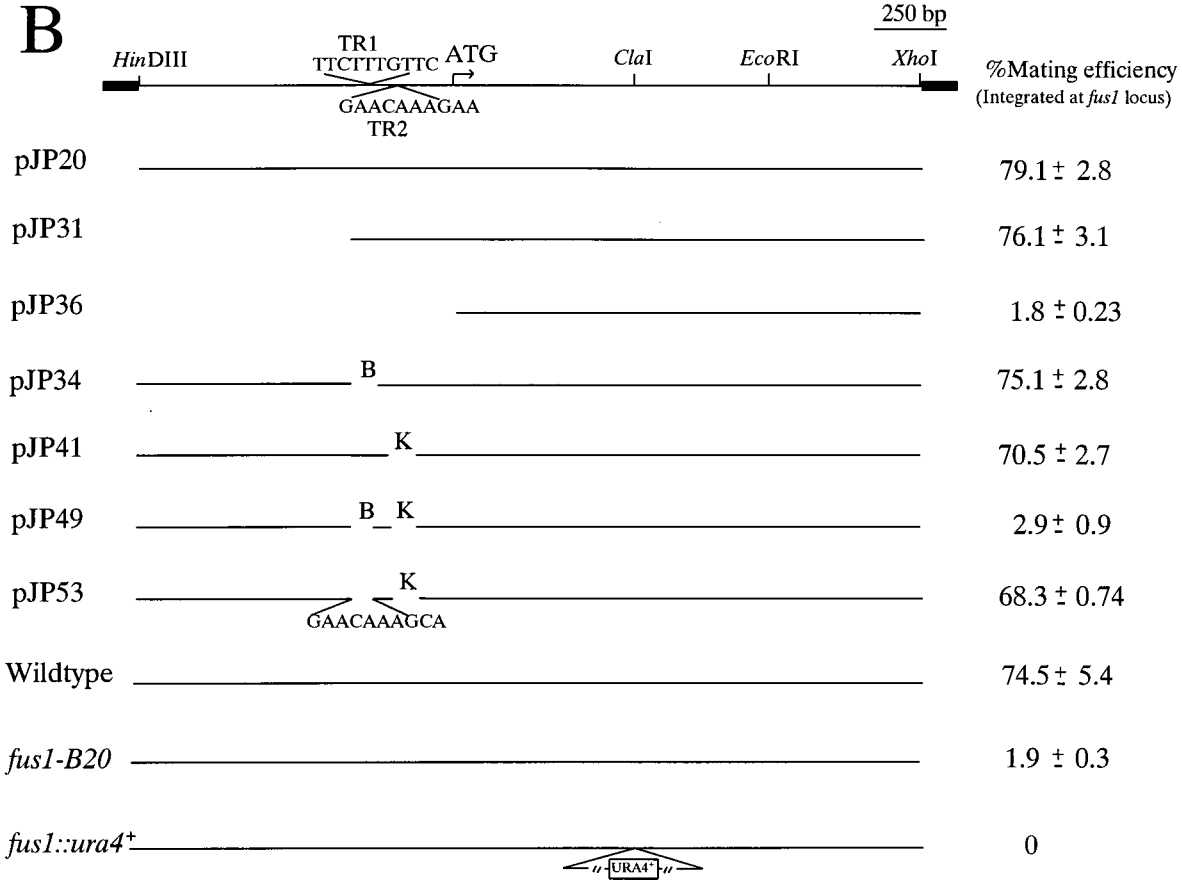

B
250 bp
HinDIII
TR1
TTCTTTGTTC
ATG
ClaI
EcoRI
XhoI
GAACAAAGAA
TR2
%Mating efficiency
(Integrated at fus1 locus)
pJP20
79.1 ± 2.8
pJP31
76.1 ± 3.1
pJP36
1.8 ± 0.23
pJP34
B
75.1 ± 2.8
pJP41
K
70.5 ± 2.7
pJP49
B K
2.9 ± 0.9
pJP53
K
GAACAAAGCA
68.3 ± 0.74
Wildtype
74.5 ± 5.4
fus1-B20
1.9 ± 0.3
fus1::ura4+
URA4+
0

FIG. 8. Deletion analysis of the *fus1* 5′-upstream region. (A) Strategy for integration of promoter-mutated plasmids, at the *fus1* locus, in EG 325 (*h*$^-$ *ura4-D18*). (B) The two TR boxes in the *fus1* promoter are shown (TR1 and TR2). Replacement of the TR boxes by restriction sites was done by PCR. K, *Kpn*I; B, *Bam*HI. The TR box inserted in pJP53 is identical to one from the *mat1-Pm* promoter. Wild type, EG 282; *fus1-B20*, EG 382; *fus1*::*ura4*$^+$, EG 439. The mating efficiencies of the various constructs were calculated as described in Materials and Methods. Values represent means of at least three separate trials ± standard deviations. (C) Phenotypes of selected integrants.

The initial breakdown of the wall and membrane is presumably accomplished by enzymatic activities. From the genetic and physiological behavior of the *fus1* mutant, we conclude that its gene product acts at the contact zone between mating pairs to regulate or participate in cell wall degradation, reorganization, and plasma membrane fusion, and this function is usually activated in both mating types. The phenotype conferred by the null mutation is almost identical to that of the original *fus1* mutant (5). The slightly leaky phenotype of this mutant is probably due to some residual activity, since the disrupted allele seems to be tight (Fig. 8B). The viability of the haploid *fus1*::*ura4* cells demonstrates that the *fus1* gene is not essential.

In fission yeast cells, all manifestations of cellular mating type are repressed during vegetative growth. Depletion of certain nutrients, most notably the nitrogen source, provokes both conjugation and sporulation (11). It was therefore of interest to investigate whether transcription of the *fus1* gene is under nutritional regulation (Fig. 6). As expected, the *fus1* transcript was not induced in vegetative cells of an h^{90} strain, but after nitrogen starvation, the expression of the transcript was greatly stimulated. We found that heterothallic strains failed to induce the *fus1* transcript to the level seen in the homothallic strain, which suggests that the major induction requires a pheromone signal (Fig. 6A). Consistent with this, we found that addition of either P factor to *M* cells or M factor to *P* cells causes a strong induction of the *fus1* transcript (Fig. 6B). Previously, it has been shown that the *mat1-Pm* gene is induced by M factor (49), whereas the *sxa2* gene (27) and the three *mfm* genes (30) are induced by P factor. Thus, *fus1* is the first example of an *S. pombe* gene that is induced by both P factor and M factor.

Consistent with *fus1* being pheromone controlled, we found that mutations in genes involved in transmission of the pheromone signal abolished transcription (Fig. 7). Expression of the *S. cerevisiae FUS1* gene is also activated by mating pheromones and requires an intact pheromone response pathway (22). *S. cerevisiae FUS1* transcription is regulated by the transcription factor STE12, which binds to PREs in promoters of pheromone-controlled genes (10). It has been shown that deletion of four PREs in the *S. cerevisiae FUS1* promoter abolished expression (22). From the present data, it is not obvious how the TR box is involved in the control of genes transcribed in response to a pheromone signal in *S. pombe*. Point mutations in a TR box upstream of *mat1-Pm* prevented expression of the gene (1), but perhaps the TR box is only required to transmit the signal of nitrogen starvation. Expression of Ste11, the transcription factor which binds to the TR box (59), is strongly induced in response to nitrogen starvation. Whether Ste11 can be directly activated by the pheromone pathway or whether this pathway functions through another yet unidentified transcription factor is unsettled at present. Mutation of the *ste11* gene prevents *fus1* transcription (Fig. 7), but this could be an indirect effect of other components in the signal transduction pathway not being expressed. However, the fact that deletion of the two TR boxes in the *fus1* promoter abolished expression (Fig. 8) strongly suggests that the *ste11* gene product is directly required for *fus1* expression. The results presented in Fig. 8 indicate that one TR box is sufficient to maintain wild-type activity of the *fus1* gene even if the TR box is taken from another gene. Furthermore, deletion analysis of the *mat1-Pm* promoter has shown that removal of both TR elements is required to see an effect on expression (1). In the *fus1* 5′-upstream region, the TR box seems to function in both orientations (Fig. 8B). One could argue that a similar situation exists in the *matP* promoter. *mat1-Pc* and *mat1-Pm* are transcribed in opposite directions, and the same region functions as promoter for both genes (28). The *Pc* transcript is strongly induced by nitrogen starvation, whereas *Pm* in addition needs a pheromone signal (28, 49). In this promoter, two TR boxes are oriented in the same direction (TTCTTTGTTY relative to *Pm* expression). A mutation in *ste11* abolished transcription of both genes (59), suggesting that the TR boxes function as PAACAAAGAA when mediating *Pc* expression.

Despite the similar phenotypes of the *fus1* mutants in *S. cerevisiae* and *S. pombe*, these proteins may perform different functions in related pathways. The identification of six *FUS* complementation groups in *S. cerevisiae* (17, 34, 40, 62) suggests that several genes are involved at this stage of cell fusion. *S. cerevisiae* Fus1 is a membrane protein which localizes to the site of cell fusion, the projection tip (40, 63). The sequence of *S. pombe fus1* does not indicate that it encodes a membrane

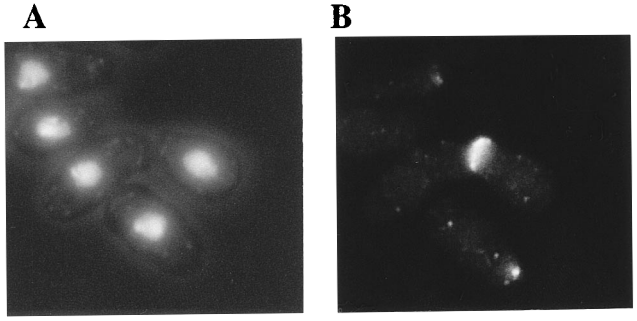


FIG. 9. Indirect immunofluorescence microscopy of cells expressing the triple HA-Fus1 construct from the *fus1* promoter in single copy with primary antibodies against the HA tag. (A) DAPI phase staining. (B) HA-Fus1 staining. A fluorescence signal can be seen at the projection tip.

TABLE 3. Comparison of the proline-rich sequences in *fus1* with SH3 binding sites

Protein	Sequence[a]	Amino acids	Reference reporting binding to an SH3 domain
S. pombe Fus1	**P** F K A **P P P** A **P** L	803–812	
S. pombe Fus1	**P** A **P** L **P P P** A **P P**	809–818	
3BP-1	A **P** T M **P P P** L **P P**	266–275	7
Dynamin	**P** A **P** G **P P P** A G		4
mSOS1[b]	E V **P** V **P P P** V **P P**	1149–1158	56
mSOS1	**P P** E S **P P** L L **P P**	1210–1219	56
m4maChr	**P P** A L **P P P P** R **P**		52
mFormin	A **P P** T **P P P** L **P P**	872–881	52

[a] Binding of the sequences shown to an SH3 domain (a 50-amino-acid domain) has been demonstrated in all cases except *fus1*. The two *fus1* sequences are partially overlapping (Fig. 4). The proline residues (P) are in boldface.
[b] m, mouse.

protein, and it has no obvious signal sequence, in contrast to budding yeast *FUS1*.

The DNA sequence of the *fus1* gene revealed a 1,372-amino-acid ORF (Fig. 4) with no significant homology to other proteins in the databases. Hence, the structure of *fus1* does not suggest that it encodes any known enzymatic activity (e.g., a glucanase), but one cannot exclude the possibility that it encodes a yet unknown enzymatic activity. Another possibility is that the *fus1* gene product is involved in the process of controlling degradation of the cell walls during mating. Constitutive expression of the functional HA-tagged Fus1 protein did not cause cell death, indicating that the presence of Fus1 protein in itself does not directly cause cell wall degradation. This conclusion is also supported by the fact that heterothallic strains will induce *fus1* already when exposed to pheromone (Fig. 6B). Rather, it would appear that the process of cell wall degradation is triggered after cell agglutination and successful active-pair formation and that *fus1* mutants cannot receive this signal. Consistent with this idea is the observation that *fus1* mutants fail to switch off the cell elongation process and hence produce horseshoe-shaped prezygotes (Fig. 2C).

Comparative studies of membrane proteins have revealed that *S. cerevisiae* Fus1 contains an Src homology 3 (SH3) domain (54). SH3 domains are small, 55- to 70-amino-acid protein motifs involved in protein-protein interaction through recognition of proline-rich sequences (7, 69). A large number of SH3-containing proteins participate in the control of cytoskeletal organization (39), suggesting that these domains are involved in regulating the interaction of signal molecules with the cytoskeleton. SH3-mediated interaction with membrane proteins has also been observed (55). Sequence analysis of *S. pombe fus1* revealed the presence of a proline-rich motif (Fig. 4). Results from comparison of this sequence with other proline-rich sequences which have been shown to interact with an SH3 domain are summarized in Table 3. Perhaps *S. pombe* Fus1 and *S. cerevisiae* Fus1 regulate cell wall degradation during mating by interaction through, respectively, a proline-rich region (the SH3 binding sites) and an SH3 domain. One could then imagine that the other interacting proteins, containing the opposite part of the SH3 binding complex, could be the products of another *fus* gene. Our finding that *S. pombe* Fus1 appears to be located at the projection tip (Fig. 9) is compatible with this idea. Whether one of the five other *S. cerevisiae* genes contains a proline-rich region is not yet known.

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ADDENDUM IN PROOF

We have discovered that the Fus1 protein has sequence similarity to the *S. cerevisiae* bud site selection protein Bni1 (GenBank accession number L31766) and the *Drosophila* cytokinesis protein diaphanous (GenBank accession number U11288).

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