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Genetics

Cloning of the *STE5* gene of *Saccharomyces cerevisiae* as a suppressor of the mating defect of *cdc25* temperature-sensitive mutants

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ABSTRACT **The *STE5* gene of *Saccharomyces cerevisiae* was cloned using a screening procedure designed to isolate genes of the *S. cerevisiae* pheromone response pathway. We screened a yeast genomic high-copy-number plasmid library for genes that allow mating of *cdc25*^{ts} mutants at the restrictive temperature without affecting the cell-cycle-arrest phenotype. One of the genes cloned was identified by genetic analysis as *STE5*. *STE5* encodes a predicted open reading frame of 916 amino acids and exhibits significant homology to Far1 protein. RNA blot analysis reveals that *STE5* gene transcription is regulated by the mating type of the cell and depends on an intact pheromone-response pathway.**

In addition to their growth defects, many temperature-sensitive (ts) cell-division-cycle mutants fail to mate at the restrictive temperature, presumably because they arrest growth at a point in the cell cycle that is incompatible with mating (1). Among the cdc mutants that also show mating defects are *cdc25* (2) and *cdc35* (3). *CDC25* encodes a Ras GDP/GTP exchange factor necessary for Ras-mediated activation of adenylyl cyclase (4–8), whereas *CDC35* encodes the cyclase itself (9). Both *cdc25* and *cdc35* ts mutants arrest at the restrictive temperature as unbudded cells in G_1 (10).

When exposed to mating pheromone of the opposite cell type, wild-type cells arrest growth at a point in G_1 called Start and undergo a morphological change called "shmoo" (for recent reviews of the yeast pheromone response, see refs. 11–13). Upon shift to the restrictive temperature, *cdc25*^{ts} and *cdc35*^{ts} cells lose the ability to shmoo in response to pheromone as well as the ability to mate. The failure of *cdc25*^{ts} and *cdc35*^{ts} strains to mate at the restrictive temperature could be interpreted as evidence that they arrest at a point in G_1 before the pheromone-induced arrest point Start. However, we thought that the growth and mating defects of *cdc25*^{ts} and *cdc35*^{ts} mutants might represent separate phenotypes. We therefore screened a yeast high-copy-number plasmid library for genes that would allow mating of *cdc25*^{ts} cells at the restrictive temperature without allowing growth.¶

MATERIALS AND METHODS

Yeast Medium and Genetic Techniques. YPD medium contained 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose. The -ura medium contained 0.17% yeast nitrogen base without amino acids and without ammonium sulfate, 0.5% ammonium sulfate, and 2% glucose, supplemented with 0.2% Casamino acids, adenine at 40 μg/ml, and tryptophan at 20 μg/ml. Standard yeast genetic techniques were used (14, 15). Yeast transformations were done by the lithium acetate procedure (16). Plasmids were recovered from yeast by using glass beads (17) and were transfected into *Escherichia coli* for further analysis. DNA was manipulated by standard techniques (18).

Yeast Strains. Yeast strains used in this work are described in Table 1. The diploid used to test linkage between the functional region of p2-1 and *STE5* was created by crossing strain SM1414 with strain βWT-5 at 23°C, the permissive temperature for SM1414, whereas the plasmid pC33-2-1-PN of strain βWT-5 was subsequently lost from the diploid.

Growth, Mating, and Pheromone-Response Assays. Growth and mating were assayed by using plate assays (15). Individual colonies were patched onto YPD or -ura plates as required, grown at 23°C, and replica-plated to four fresh YPD or -ura plates, two of which were incubated at 23°C and 34°C, respectively, to test for growth, whereas the other two were grown at 23°C overnight and used to assay mating. To test mating the patches were replica-plated to fresh YPD plates and incubated for 1 hr at 23°C or 34°C, after which they were overlaid with tester strain 310 or 311, and incubation was continued at the same temperature for 20 hr. Subsequently the plates were replica-plated to SD plates to select for diploids. To assay pheromone response α-factor at 10 μg/ml was added to exponentially growing *MAT***a** cells at 23°C or upon shift of the culture to 34°C. Percent shmoo was monitored 5 hr after α-factor addition. At least 1000 cells were counted in each case.

Plasmids. Genomic inserts were cloned into YEp24 (20) for high-copy number or into the centromeric vector YCplac33 (21) for low-copy number. pFL1-6 is a YEp24-based plasmid carrying a genomic insert including *CDC25* (19). p2-1 is a YEp24-based plasmid carrying *STE5* on the genomic insert shown in Fig. 2*A*. This plasmid was isolated in this study as a high-copy-number suppressor of the nonmating phenotype of *cdc25*^{ts} at the restrictive temperature. p2-1-PN carries the 3.6-kb *Pvu* II–*Nhe* I fragment of clone 2-1 (see Fig. 2) on YEp24. pC33-2-1-PN carries the same 3.6-kb *Pvu* II–*Nhe* I fragment of clone 2-1 on YCplac33.

Genomic Deletion Plasmids. p2-1KS-C::HIS3, used to create genomic deletions of *STE5*, was constructed by cloning the 4.5-kb *Cla* I–*Cla* I fragment of the genomic insert of p2-1 into pBluescriptIIKS(+) (Stratagene), after which the 1.7-kb *Kpn* I–*Sal* I fragment of *STE5* was replaced with a 1.8-kb *Bam*HI–*Bam*HI fragment encoding *HIS3*. p4PUC::HIS3, used to create genomic deletions of *STE4*, was constructed by cloning the 1.9-kb *Eco*RI–*Eco*RI fragment of pGTSTE4-1 (22) into the *Eco*RI site of pUC19, after which the *Bst*BI–*Bst*BI fragment of *STE4* was replaced with a 1.8-kb *HIS3* fragment as above. p12KS::HIS3, used to create genomic

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Abbreviation: ts, temperature-sensitive.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. L01620).

Table 1. Yeast strains

Strain	Genotype	Source/ref.	Relevant genotype
352-5A1	*MATa cdc25-2 ade lys2 his7 ura3-52*	19	*cdc25^{ts}*
2192	*MATα cdc25-2 leu2 his4 lys2 ura3-52 ade2-101 met10 trp1 ime1::TRP1*	Laboratory stock	*cdc25^{ts}*
SM1414	*MATα ste5-1 ura3 his trp*	S. Michaelis*	*ste5^{ts}*
βWT	*MATa ura3-52 lys2 leu2 trp1-289 his3Δ-200 met GAL$^+$*	This work	**a**
βWT-α	*MATα ura3-52 lys2 leu2 trp1-289 his3Δ-200 met GAL$^+$*	This work	α
βWT-**a**/α	*MATa/MATα ura3-52/ura3-52 lys2/lys2 leu2/leu2 trp1-289/trp1-289 his3Δ-200/his3Δ-200 met/met GAL$^+$/GAL$^+$*	This work	**a**/α
βWT-4	*MATa ura3-52 lys2 leu2 trp1-289 his3Δ-200 met GAL$^+$ ste4::HIS3*	This work	*ste4Δ*
βWT-5	*MATa ura3-52 lys2 leu2 trp1-289 his3Δ-200 met GAL$^+$ ste5::HIS3*	This work	*ste5Δ*
βWT-12	*MATa ura3-52 lys2 leu2 trp1-289 his3Δ-200 met GAL$^+$ ste12::HIS3*	This work	*ste12Δ*
310	*MATα lys1*	Laboratory stock	
311	*MATa lys1*	Laboratory stock	

Isogenic **a**, α, and **a**/α strains were constructed by transforming strain βWT with a plasmid carrying *HO* and isolating strains of each mating type after allowing the transformants to lose the *HO* plasmid. Strains βWT-4, βWT-5, and βWT-12 were constructed by introducing *STE4*, *STE5*, and *STE12* deletions into strain βWT by gene replacement with plasmids p4PUC::HIS3, p2-1KS-C::HIS3, and p12KS::HIS3 for deletion of *STE4*, *STE5*, and *STE12*, respectively. All deletion mutations were confirmed by Southern blot analysis (data not shown).
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deletions of *STE12*, was constructed by cloning the *Sac* I–*Bam*HI and *Xba* I–*Xba* I fragments of *STE12* (23) into pBluescriptKS(+), leaving a deletion of the 0.6-kb *Bam*HI–*Xba* I fragment of *STE12*, followed by insertion of the 1.8-kb *HIS3* fragment in place of this deletion, as above.

Nucleotide Sequence Analysis. DNA sequencing was done on both strands by the dideoxynucleotide chain-termination method (24) using the Sequenase version 2.0 kit (United States Biochemical). Templates for sequencing were prepared by cloning genomic DNA fragments into Bluescript plasmids (Stratagene) and creating nested deletions by digesting the genomic inserts with *Exo* III using the Erase-a-Base kit (Promega). Single-stranded DNA was prepared by transforming TG1 cells with the deleted plasmids followed by superinfection with R408 helper phage (Stratagene). Sequencing reaction products were run on Long Ranger (AT Biochem, Malvern, PA) gel using the recommended procedure. Sequence analysis was done with the aid of the Genetics Computer Group sequence analysis software package, version 7.0 (25).

RNA Blot Analysis. Total RNA was isolated by using a glass-bead preparation method (26), electrophoresed on a Mops/formaldehyde-1% agarose gel for 18 hr at 40 mV and blotted onto a nitrocellulose membrane (18). ^{32}P-labeled probes were generated by using a random priming kit (Boehringer Mannheim).

RESULTS

A Genetic Screen for High-Copy-Number Suppressors of the *cdc25^{ts}* Mating Defect. A yeast genomic library constructed in the high-copy-number vector YEp24 (26) was introduced into the *cdc25^{ts} MATα* strain 2192. Approximately 12,000 Ura$^+$ transformants were patched onto -ura plates and tested for growth and mating at 23°C and 34°C, as shown in Fig. 1. For further study we selected transformants that could form diploids at the restrictive temperature but were still unable to grow at this temperature (Fig. 1). An additional 10 transformants displayed the ability to grow as well as mate at the restrictive temperature; these were not analyzed further. Plasmids were recovered from transformants that showed cosegregation of the plasmid with the ability to mate at 34°C. A total of 20 different plasmids were isolated, introduced into the *cdc25^{ts} MATa* strain 352-5A1, and tested for their ability to allow shmoo formation in response to α-factor at the restrictive temperature. Of these, plasmid p2-1 was identified as the most effective suppressor of the mating defect of *cdc25^{ts}* mutants. Although *cdc25^{ts}* mutants cannot form shmoos at the restrictive temperature (ref. 2; Table 2), plasmid p2-1 allowed 21% shmoo formation at 34°C (Table 2).

Characterization of Plasmid p2-1. Plasmid p2-1 carries a 13-kb genomic insert shown in Fig. 2*A*. To identify the functional region of the insert, subclones were constructed and tested for their ability to allow shmoo formation of *cdc25^{ts}* mutants in response to α-factor at 34°C (Fig. 2*B*). Plasmid p2-1-PN, in which all insert sequences left of the *Pvu* II site were removed, retained the ability to suppress the ts mating defect of *cdc25^{ts}* mutants, whereas all plasmids in which sequences right of the *Pvu* II site were removed retained only partial or no activity. We therefore defined the functional region of the insert as those sequences between the *Pvu* II site and the right end of the insert, as shown in Fig. 2*A*.

A genomic deletion of most of the functional region of p2-1 resulted in a sterile phenotype in haploid cells of both mating types, indicating that the deleted gene is required for mating.

The p2-1 genomic insert was mapped to chromosome IV by chromosome blotting (ref. 27; data not shown). The location within chromosome IV was determined by hybridization to a set of phage λ clones harboring previously mapped *Saccharomyces cerevisiae* genomic inserts (27, 28). The *Sal* I–*Cla* I fragment of p2-1 hybridized to a phage mapped to the right arm of chromosome IV, 20 kb from the *ARO1* locus, between *ARO1* and the centromere.

Plasmid p2-1-PN Encodes *STE5*. Standard genetic analyses showed that the *Pvu* II–*Nhe* I fragment of p2-1 encodes *STE5*,

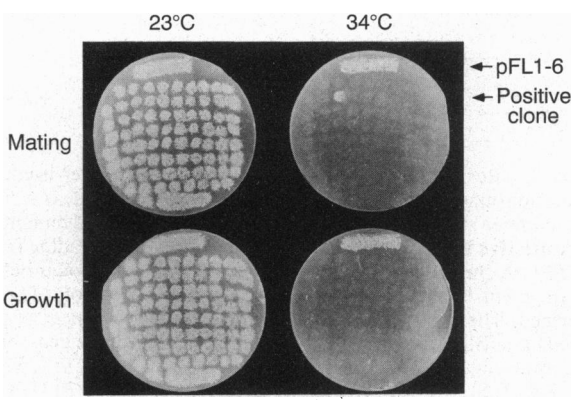

FIG. 1. A genetic screen for high-copy-number plasmids that allow mating, but not growth, of *cdc25^{ts}* mutant strain 2192 at 34°C. Depicted is a representative set of plates showing isolation of one positive clone. Ura$^+$ transformants were grown as patches on a -ura plate at 23°C; then mating and growth were tested by using plate assays as described.

Table 2. Response to α-factor of *cdc25*^{ts} mutant cells, rescued by CDC25 or by clone p2-1

Plasmid*	Shmoo, % 23°C	34°C
YEp24	55	0
pFL1-6	74	78
p2-1	60	21

Shmoo formation in response to pheromone was assayed at the permissive and restrictive temperatures as described in text. Cells were strain 352-5A1 transformed with indicated plasmids.
*YEp24, control vector; pFL1-6, *CDC25* on YEp24; p2-1, clone 2-1 isolated in the genetic screen for high-copy-number suppressors of the *cdc25*^{ts} mating defect at the restrictive temperature.

a gene previously identified in genetic screens for pheromone-resistant or sterile mutants (29, 30) and genetically mapped to chromosome IV (31). The *Pvu* II–*Nhe* I fragment of p2-1 complements the sterility of the *ste5*^{ts} strain SM1414 at the restrictive temperature, whether introduced on a high-copy-number plasmid (p2-1-PN) or on a centromeric plasmid (pC33-2-1-PN) (data not shown), suggesting that the fragment encodes *STE5* itself and not a high-copy-number suppressor of *ste5*^{ts}. Genetic linkage between *STE5* and clone p2-1 was demonstrated by tetrad analysis using a diploid created by crossing a *ste5*^{ts} strain with a strain carrying a genomic disruption of the functional region of p2-1 (see *Materials and Methods*.) The *ste5*^{ts} allele and the p2-1 genomic disruption segregated 2:2 in dissections of 36 tetrads, producing in every case two sterile spores and two spores that are ts for mating. Together, the complementation results and the linkage results show that p2-1-PN encodes *STE5*.

Sequence Analysis of *STE5*. Sequence analysis of the 3.6-kb *Pvu* II–*Nhe* I fragment of clone 2-1 revealed a putative open reading frame of 916 amino acids, flanked by 570 bp of noncoding sequence at the 5′ end and 152 bp at the 3′ end (Fig. 3). The predicted sequence of the protein does not

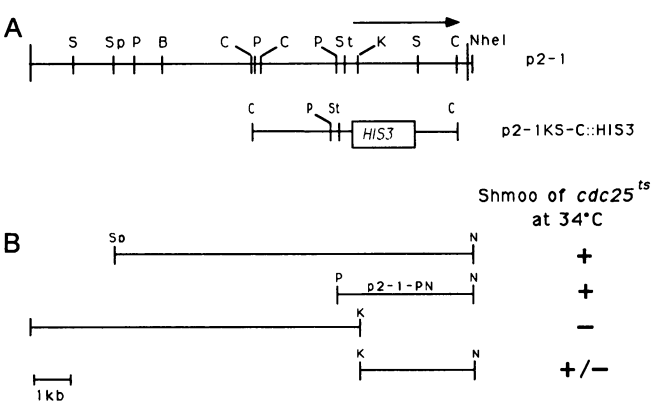

FIG. 2. Restriction map of clone p2-1 and of subclones used. (*A*) Restriction map of the genomic insert of p2-1, isolated as a high-copy-number suppressor of the mating defect of *cdc25*^{ts} mutants at the restrictive temperature. Arrow indicates the open reading frame of *STE5*. A fragment of plasmid p2-1KS-C::HIS3 is shown below; this fragment is used for creating a genomic deletion of *STE5*, as described. The solid bar indicates the *Cla* I–*Cla* I fragment of p2-1 cloned into pBluescriptKS(+). The open bar indicates the yeast *HIS3* gene, replacing the *Kpn* I–*Sal* I fragment of *STE5*. C, *Cla* I; S, *Sal* I; K, *Kpn* I; St, *Stu* I; P, *Pvu* II; B, *Bam*HI; Sp, *Sph* I. (*B*) Deletion analysis of the functional region of p2-1. Suppression of the *cdc25*^{ts} mating defect was assayed by transforming strain 352-5A1 with YEp24-based plasmids carrying the indicated inserts and testing for shmoo formation in response to α-factor at 34°C, as described in text. +, Shmoos seen in at least 20% of cells; −, shmoos not observed; +/−, partial suppression of the mating defect with few shmoos appearing after a significant delay.

suggest any known catalytic activity or structural motifs, and the only remarkable feature noted was a highly acidic domain located in the C terminus between residues 775 and 876. In this region, 30% of the residues are either aspartate or glutamate.

Analysis of the upstream noncoding sequences revealed two types of regulatory sites characteristic of genes in the pheromone-response pathway: a potential a_1–α_2 binding site, and five potential pheromone-response elements. a_1–α_2 binding sites mediate repression of haploid-specific gene transcription in diploid cells (32), whereas pheromone-response elements are required for induction of transcription in response to pheromone (33, 34). The potential a_1–α_2 site, found 190 bp upstream of the putative translation start site (Fig. 3), differs at only 2 bp from the reported consensus sequence (TCRTGTNNWNANNTACATCA) (35). The five potential pheromone-response elements are imperfect matches, where 6 out of 7 bases were found to match the consensus sequence TGAAACA (Fig. 3) (33).

A search for similar proteins in the Swiss-Prot and Protein Identification Resource data bases was done by using the BLAST program (36). Most proteins identified in the search contained acid-rich domains similar to the highly acidic C terminus of Ste5; however, none of these proteins were significantly similar to Ste5 outside of this domain, rendering the significance of the homology questionable. The only protein that displays significant homology to Ste5 outside the acid-rich region is Far1, a *S. cerevisiae* protein necessary for cell-cycle arrest in response to α-factor (37). Alignment of Ste5 and Far1 was done by using the BESTFIT program with the randomization option for analysis of statistical significance (25). Overall, Ste5 and Far1 show 44% similarity; however, only the homology between residues 170–511 of Ste5 and residues 145–491 of Far1 was statistically significant (Fig. 4). In this region Ste5 and Far1 are 23% identical and 48% similar, and the quality score for the alignment is 13 SDs higher than the average score expected between random proteins of the same amino acid content.

Regulation of *STE5* Gene Expression. RNA analysis revealed a major STE5 transcript of ≈3.5 kb (Fig. 5), which was not detected in an isogenic strain carrying a genomic deletion of *STE5* (data not shown). The transcript is haploid specific (Fig. 5, lanes 3–5), as predicted by the presence of an a_1–α_2 binding site in the 5′ noncoding region of *STE5*. However, the transcript is present at unequal levels in isogenic *MAT***a** and *MAT*α strains, with ≈10-fold lower levels found in *MAT*α cells (Fig. 5, lanes 3 and 4). The reason for differential expression in **a** and α cells is unclear, but similar differences have been seen in other haploid-specific genes (37–39). Isogenic *MAT***a** strains carrying deletions in either *STE4* or *STE12* showed ≈10-fold reduced levels of STE5 transcript (Fig. 5, lanes 1 and 2), as is seen for other mating-specific genes, such as *STE2*, *STE3* (40), and *FAR1* (37).

An additional STE5 transcript of ≈2 kb was also observed. This transcript is specific to *STE5* because it is not seen in an isogenic *STE5*-deleted strain (data not shown). However, the smaller transcript does not appear to be regulated because it was present at similar levels under most conditions tested. It is therefore likely that the larger transcript encodes the full *STE5* open reading frame, whereas the exact nature of the smaller transcript remains to be determined.

DISCUSSION

We have screened a high-copy-number genomic library for plasmids that allow mating but not growth of *cdc25*^{ts} mutants at the restrictive temperature. This screening procedure yielded a mating pathway gene identified as *STE5*.

STE5 had been identified in genetic screens for pheromone-resistant or sterile mutants (29, 30). Its molecular function is

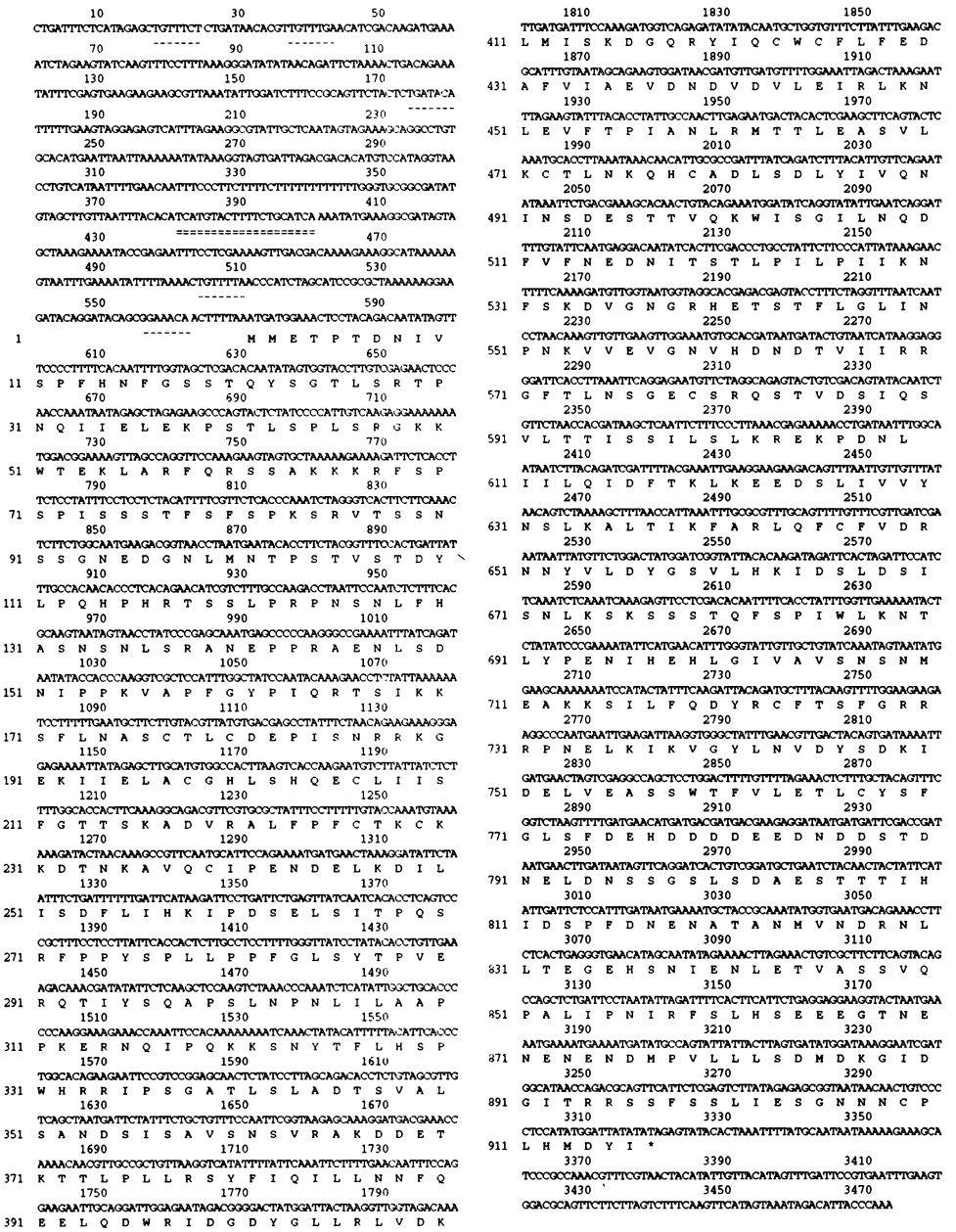

FIG. 3. DNA sequence of *STE5*, and the putative protein translation product. Sequence of the *Pvu* II–*Nhe* I fragment of clone p2-1. One hundred and forty base pairs of plasmid sequences between the end of the genomic insert and the *Nhe* I site were omitted. Sequences marked in the 5′ noncoding region are as follows: a potential a_1–α_2 binding site is double-underlined, and imperfect (6/7) matches to the pheromone-response element consensus are single-underlined.

unknown, but genetic analyses suggest that it acts in the mating pathway downstream of the G protein encoded by *SCG1/GPA1*, *STE4*, and *STE18* (41, 42) and upstream of the protein kinases encoded by *STE7*, *STE11*, *FUS3*, and *KSS1* (43–45). We have shown *STE5* to be transcribed in a manner typical of mating-specific genes: it is haploid-specific and depends on an intact pheromone-response pathway for transcription at normal levels.

The sequence of *STE5* reveals nothing concerning its biochemical function. The predicted protein contains an

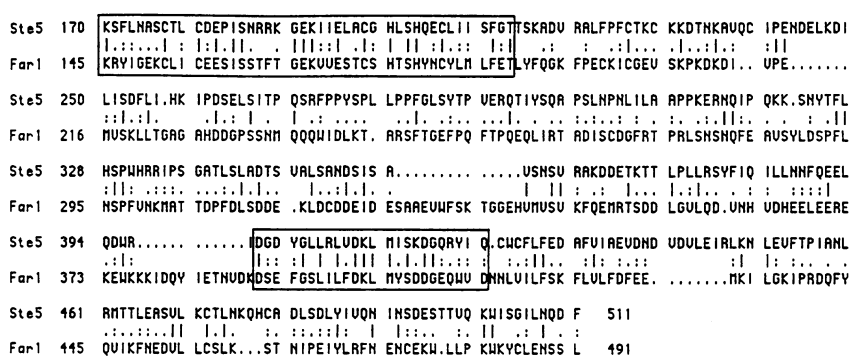

FIG. 4. Alignment of the homologous regions of Ste5 and Far1. The BESTFIT program was used to optimize alignment of the proteins (25). Regions of most significant homology are boxed.

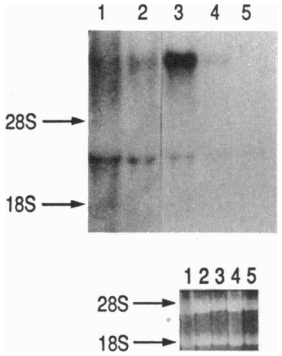

FIG. 5. RNA blot analysis of the *STE5* transcript. Total RNA was isolated from the strains listed below and subjected to RNA blot analysis with the *Kpn* I–*Sal* I fragment of *STE5* used as a probe. Lanes: 1, strain βWT-12 (*ste12Δ*); 2, strain βWT-4 (*ste4Δ*); 3, strain βWT; 4, strain βWT-α; 5, strain βWT-a/α.

acid-rich region resembling the acid blobs found in many transcription factors (46); however, many proteins that are not known to be transcription factors also contain acid-rich regions; furthermore, Ste5 shows no homology to any known transcription factor outside the acid-rich domain. The protein also contains several putative phosphorylation sites, suggesting that it may be regulated at the phosphorylation level.

The only known protein significantly homologous to Ste5 is Far1, another protein involved in the yeast pheromone response. Far1 is necessary for cell-cycle arrest in response to pheromones and contributes to mating efficiency; however, it is not essential for other aspects of the pheromone response, such as activation of pheromone-inducible gene expression (37). Interestingly, the N terminus of Far1, which exhibits significant homology to Ste5, seems to be the domain required for cell-cycle arrest (37). In contrast to Far1, Ste5 is absolutely required for pheromone-induced transcription of *FUS1* (ref. 47; data not shown); however, it is tempting to speculate that Ste5 may also play a role in cell-cycle arrest in response to pheromone.

STE5 overexpression suppresses the mating defects of both *cdc25*^{ts} (this study) and *cdc35*^{ts} strains (data not shown). The mechanism by which *STE5* overcomes the mating defect of these strains is still unclear. The failure of *cdc25*^{ts} and *cdc35*^{ts} strains to mate at the restrictive temperature could be interpreted as evidence that they arrest at a point in G_1 prior to the pheromone-induced arrest point Start. Alternatively, the mating defect may indicate that cAMP is in some way required for mating. We have not yet succeeded in distinguishing between these two possibilities. Overexpression of *STE5* might cause some cells in a population to arrest at Start, thereby protecting them from arrest at the cdc25/35 arrest point and allowing them to mate. Alternatively, *STE5* overexpression might allow cells to mate in spite of arrest of the cell cycle early in G_1. Finally, *STE5* overexpression may overcome some putative negative regulation of the mating pathway imposed by the lack of cAMP.

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