Multiple Regulation of STE2, a Mating-Type-Specific Gene of Saccharomyces cerevisiae

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The Saccharomyces cerevisiae STE2 gene, which is required for pheromone response and conjugation specifically in mating-type a cells, was cloned by complementation of the ste2 mutation. Transcription of STE2 is repressed by the MATa2 gene product, so that the 1.4-kilobase STE2 RNA is detected only in a or mata2 strains, not in a or a/α cells. However, STE2 RNA levels are also increased by the mating pheromone α-factor and decreased in strains bearing mutations in the nonspecific STE4 gene. Regulation of STE2 expression in a cells is therefore achieved by several mechanisms.

Mating-type expression in the yeast Saccharomyces cerevisiae is an important model for development and regulation in eucaryotic cells (reviewed in references 14, 38, and 49). Two alleles, MATa and MATα, determine the mating type of cells as a or α, respectively. Mating between a and α haploid cells results in a third cell type, the a/α diploid, which is unable to mate but can be induced to undergo meiosis and sporulation. To establish the mating type of a haploid cell, certain a- or α-specific characteristics must be expressed. For example, a cells secrete the mating pheromone α-factor and can respond to the pheromone α-factor, whereas a cells produce a-factor, are sensitive to α-factor, and secrete Barrier activity, a protein that reverses the effects of α-factor on a cells (reviewed in references 14, 49, and 58).

MacKay and Manney (30) proposed that the MAT alleles code for regulatory proteins, which control the expression of genes (or their products) that are unique to the MAT locus and that are necessary for establishing cell type and for conjugation. These unlinked genes (denoted STE for the sterile mutant phenotype) were identified by the isolation and subsequent genetic analysis of various nonmating mutants (13, 29, 30, 50; L. C. Blair, Ph.D. thesis, University of Oregon, Eugene, 1979; J. R. Rine, Ph.D. thesis, University of Oregon, Eugene, 1979). Some of the STE gene products, i.e., STE4, STE5, STE7, STE11, and STE12, were shown to be necessary for mating in both cell types. Others, however, are cell type specific: a cells require the STE2, STE6, and STE14 gene products for mating, whereas mutations in the STE3, STE13, KEX2, and TUP1 genes block mating only in a cells. The last two have pleiotropic phenotypes that extend beyond their mating defects (26, 27) and therefore are not considered conjugation-specific genes.

A hypothesis for the regulation of the mating-type-specific functions has been proposed (53) in which MATα codes for two regulatory proteins, α1 and α2, in a cells. The α1 protein acts as a positive regulator for the expression of α-specific genes, and α2 is a negative regulator that blocks the expression of α-specific genes. In a cells, MATα codes for only one known functional product, α1, which has no influence on mating but is required for sporulation in a/α diploids. The α1-α2 hypothesis predicts that the expression of at least some mating-type-specific genes should be controlled by the MATα locus. Although this regulation could be transcriptional or posttranscriptional, the structural genes for α-factor and a-factor have been cloned and demonstrated to be transcribed only in a and α cells, respectively (4, 25, 47). In contrast, the STE13 gene product, which is required for mating only in α cells, is produced in all three cell types; STE13 apparently encodes a diaminopéptidase that is involved in processing mature α-factor (22) and is not a mating-type-specific function.

In this paper we describe the cloning of a putative a-specific gene, STE2, which has been proposed to encode the α-factor receptor on the surface of a cells (13, 18, 30). We have demonstrated that STE2 is transcribed only in a cells and that its transcription is repressed by the MATα2 product, activated by the STE4 gene product, and stimulated by exogenous α-factor.

(Part of this work was reported at the 11th International Conference on Yeast Genetics and Molecular Biology, Montpelier, France, 1982.)

MATERIALS AND METHODS

Strains and genetic methods. The S. cerevisiae strains used are listed in Table 1. Genetic methods and media used were described previously (29, 36): YEPD is a rich growth medium; MV is a chemically defined, minimal glucose medium; SC is MV with nutritional supplements to satisfy the growth requirements of the auxotrophic strains listed in Table 1; SC-leu lacks only leucine; SC-leu was supplemented with 1 M sorbitol. Temperature-sensitive mating mutants were grown at either 25°C (the permissive temperature) or 35°C (the restrictive temperature); all other strains were incubated at 30°C unless noted.

For mating tests, strains were replica plated to YEPD (or to SC-leu for transformants) and cross-streaked with mating tester strains JJ-1A and JJ-1C. After the strains had grown for approximately 24 h, the plates were replica plated to MV which would support growth only of diploids formed by mating. After another 24 h of incubation, growth of diploids indicated the mating type, as well as the ability or inability to mate, of the strains tested. For the screening of transformants for those bearing the STE2 gene, transformant colonies from regeneration agar were resuspended and plated on SC-leu at approximately 1,000 cells per plate. The resulting

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colony plates were replica plated to prewarmed lawns of strain JJ-1C on SC-leu, and the mating plates were incubated for 24 h before being replica plated to MV (selective for diploids).

Plasmids. *Yeast-Esherichia coli* shuttle vector YEp13 is derived from pBR322 (3) and carries the *S. cerevisiae* chromosomal LEU2 gene and the origin of replication from the *S. cerevisiae* 2μ plasmid (5). The shuttle vector pUC12 was obtained from M. Hansen, Novo Industri A/S, Bagsvaerd, Denmark; it contains these same yeast sequences in *E. coli* plasmid pUC12 (provided by J. Messing, University of Minnesota). Plasmid pH3 (provided by L. Bell, ZymoGenetics) was constructed by isolating a 3-kilobase (kb) BglII fragment that contains the *S. cerevisiae* LEU2 gene from plasmid YEp13. The BglII ends were blunt ended with DNA polymerase I (Klenow fragment), ligated with HindIII links, digested with HindIII, and inserted into pUC12.

Transformation. *E. coli* RR1 (3) and JM83 (33) cells were transformed as described by Bolivar et al. (3). Yeast strains were transformed by a modification (28) of the method of Begg (2). Integrants were obtained by transformation with linearized plasmid DNA (42) digested at a unique site within the cloned fragment or with a linear chromosomal fragment.

Preparation of DNA and RNA. Plasmid DNA from *E. coli* was isolated by the method of Ish-Horowicz and Burke (17). Plasmid DNA from yeast for transformation into *E. coli* was usually isolated as described by Nasmyth and Reed (40), although *E. coli* transformants were not obtained from all preparations that were demonstrated to contain plasmid. In these cases, the method of Agas was used to prepare plasmid from yeast (M. Cortelyou and V. L. MacKay, unpublished data); a 10-ml SC-leu culture was chilled on ice for 30 min and centrifuged, and the cells were washed in cold distilled water and suspended in 0.2 ml of SCE (1 M sorbitol, 0.1 M sodium citrate [pH 5.8], 10 mM EDTA) containing 0.8 mg of Zymolyase 5000 (KBR A/S, Bagsvaerd, Denmark) (49). Incubation for 30 min at 37°C was followed by addition of 0.15 ml of STE (10% sodium dodecyl sulfate, 0.1 M Tris hydrochloride [pH 9.0], 10 mM EDTA), rapid mixing, and immediate boiling for 3 min. The lysis mixture was cooled on ice, 0.15 ml of 5 M potassium acetate (pH 5.6) was added, and the mixture was left on ice for 30 to 60 min before centrifugation for 5 min. For precipitation of DNA, 0.5 ml of 5 M ammonium acetate and 1 ml of isopropanol were added to the supernatant, which was then kept at −20°C for 15 min or longer. After centrifugation for 3 min, the precipitate was washed with 70% ethanol, dried in vacuo, and dissolved in 50 to 100 μl of TE (10 mM Tris hydrochloride [pH 8], 1 mM EDTA). For further purification, the DNA solution was digested with DNase I in 50 mM ammonium acetate, precipitated as described previously (54), or an equivalent volume of 95% ethanol; the treated cultures were then incubated for the time indicated for or approximately one generation time before isolation of RNA.

Electrophoresis, transfers, and hybridizations. Electrophoresis of DNA fragments through agarose gels, subsequent transfer of denatured DNA fragments to nitrocellulose filters, and hybridization to nick-translated probes were done as described previously (35). Electrophoresis of RNA samples, transfers to nitrocellulose, and hybridizations were done as described by Thomas (57), except that deoxynucleate was omitted from the hybridization solutions. Nick translations were done essentially as described by Rigby et al. (44). Radiolabeled single-stranded probes in bacteriophage M13 were prepared by the method of Hu and Messing (16).

### TABLE 1. Yeast strains used

<table>
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<tr>
<th>Strain</th>
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<td>YGSC*</td>
</tr>
<tr>
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<td>a arg1 thr1</td>
<td>M. Jagadish</td>
</tr>
<tr>
<td>JJ-1C</td>
<td>a arg1 thr1</td>
<td>M. Jagadish</td>
</tr>
<tr>
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<td>a ade2 adeX his4 leu3 ural3 gal2</td>
<td>15</td>
</tr>
<tr>
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<td>a leu2-3,112 his3-11,15 trpl-289 ural3-52</td>
<td>G. McKnight</td>
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<td>his6 leu1 met1 trpl5 can1 gal2</td>
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<td>a leu2-3,112 gal2 barl</td>
<td>T. R. Manney</td>
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<td>This work</td>
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* YGSC, Yeast Genetics Stock Center, Berkeley, Calif.
Quantitation of autoradiograms. The autoradiograms were scanned lane by lane in a Beckman DU8 spectrophotometer, and relative peak areas were normalized by comparison with one lane of each autoradiogram set arbitrarily as one. Since the intensity of some of the autoradiogram bands is beyond the linear range, the values given should be considered only semiquantitative.

DNA sequencing. Restriction fragments were subcloned into derivatives of E. coli bacteriophage M13, and single-stranded template was prepared after transfection of E. coli JM101 or JM103 (33). Sequencing was done by the dideoxy method of Sanger et al. (46).

Enzymes. All restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories. DNase I and pancreatic RNase were from Worthington Diagnostics. DNA polymerase I was from Boehringer-Mannheim Biochemicals. Enzymes were generally used as recommended by the suppliers.

RESULTS

Isolation of DNA fragments that complement the ste2 mutation. Strains XH6-10B and XH9-5C4 cannot mate because of the ste2 mutation and were used as recipients for transformation with a clone bank of total yeast genomic fragments in vector YEpl3 (41). Leu+ transformants were screened for the ability to mate, as described in Materials and Methods. Approximately 15,000 original transformant colonies were screened, and six colonies were identified that had acquired the ability to mate. Altogether, these harbored five different plasmids that complemented the ste2 mutation. The common region, presumably containing the STE2 gene, is 2.6 kb long (Fig. 1); this localization was subsequently confirmed by the ability of a PstI-BamHI fragment from pAH1 subcloned into pUC12 (plasmid pMT411) to complement the ste2 mutation.

Complementation alone is not sufficient proof that the cloned gene is actually STE2, since overexpression of one gene might complement a mutation in another gene (28, 48). Therefore, plasmids pAH2 and pAH4 were integrated into the genome of XH6-10B (a ste2) by transformation with a linearized form (42) after digestion with BamHI at the unique site in each plasmid (Fig. 1). Southern hybridization with EcoRI-digested genomic DNA from wild-type strains showed only one band, at approximately 10 kb, that hybridized to a fragment containing the 2.6-kb common region. Integration of pAH2 and pAH4 into the chromosome split the 10-kb genomic EcoRI fragment into two bands: 12.5 and 7 kb for integration of pAH2 and 9.5 and 4.1 kb for integration of pAH4 (data not shown). The appearance of the two new bands demonstrated that the integration occurred at the chromosomal location of the cloned gene. Tetrads analysis of these integrants proved that the cloned gene is STE2. The integrants were crossed with strain XG41-14B (α), and the resulting diploids were sporulated and subjected to tetrad analysis. In the 76 tetrads analyzed, no nonmating spores were found, indicating that the integration occurred within 0.65 centimorgan of the ste2 mutation in XH6-10B. Although LEU2 is normally linked to and between HIS4 and MAT, in the integrants LEU2 was unlinked to both MAT and HIS4 (data not shown). Additional mapping (9) has assigned the STE2 gene to chromosome VI (data not shown).

Identification of the STE2 transcript. When the PstI-BamHI fragment from pAH1 was used to probe RNA samples from wild-type a strains, only a single transcript, of approximately 1.4 kb, was visible on the autoradiogram even after prolonged exposure (see, e.g., Fig. 2B, lane 1). However, in other experiments with the PstI-HindIII or PvuII-PvuII fragment of pAH1 as probe, a minor transcript (approximately 1 kb) was occasionally seen (see Fig. 3 and 4). The 1.4-kb RNA was identified as the STE2 transcript by gene disruption experiments (42, 45). A DNA fragment containing the yeast LEU2 gene was inserted at several sites into the PstI-BamHI fragment of pAH1 subcloned into pUC12. LEU2 was inserted at the SalI site (pZV66) and in both orientations at the rightward HindIII site in the fragment (pZV64 and pZV65) (Fig. 2A). LEU2 was also inserted between the two HindIII sites, replacing the approximately 0.4-kb HindIII-HindIII fragment of the STE2 fragment (pZV63). The resulting plasmids were then digested with PstI and BamHI and used to transform yeast strain XP635-1OC (a barl) to leucine prototrophy; a majority of the transformants were expected to have the disrupted STE2 fragment integrated in place of the homologous chromosomal STE2 segment (42, 45). Because the STE2 gene product is required for the a cell response to α-factor (13, 29, 30), disruption of the STE2 gene and loss of the STE2 product would confer insensitivity to α-factor. Sensitivity or
mid digest or from plasmid pMT411 were assayed for sensitivity (i.e., morphological response) to α-factor. None of those transformed with pMT411 or with the pZV64, pZV65, or pZV66 digests were resistant to α-factor, whereas six of eight transformed with the pZV63 digest had become resistant. These six are presumed to have integrated the pZV63 linear fragment in place of the chromosomal STE2 gene, thereby abolishing STE2 function because of the LEU2 insertion in the gene.

Poly(A)-enriched RNA from the transformants was probed with the intact PstI-BamHI fragment to determine whether the 1.4-kb transcript was affected by any of the gene disruptions. The 1.4-kb RNA is absent only in the pZV63 transformant (Fig. 2B, lanes 2 through 4). Similar results were obtained when total RNA from two transformants of each plasmid type was analyzed (data not shown). Since the absence of the 1.4-kb transcript corresponds to the loss of the STE2 gene function by gene disruption, this RNA is encoded by STE2.

**Regulation of STE2 transcription.** Analysis of poly(A)-enriched RNA from a variety of yeast strains showed that transcription of the STE2 gene is regulated by the mating-type locus (Fig. 3). The 1.4-kb STE2 RNA was found in α, mata1, and mata2 strains, but not in α, mata1, or α/a cells. These results support the α-a2 hypothesis (53) that α-specific genes are negatively regulated by the MATa2 gene product. To confirm MATa2 regulation of STE2 at the transcriptional level, we examined RNA samples from four different mata2 mutants grown at 23 and 36°C. Three of these are nonmaters at both temperatures, and all had the 1.4-kb band (see, e.g., Fig. 3B, lanes 5 and 6). The fourth mata2 mutant is temperature sensitive (32); at 23°C it mates as an α, and the 1.4-kb transcript was not detected (Fig. 3B, lane 4). However, at 36°C, mating is very poor (<0.1% of mating at 23°C), and a low level of the STE2 transcript was found (approximately 20% of the wild-type α at 36°C; Fig. 3B, lane 3; Table 2). Thus, the MATa2 gene product directly or indirectly represses transcription of the STE2 gene.
Poly(A)-enriched RNA samples from the nonspecific ste mutants were also probed for the STE2 transcript (Fig. 4); for all temperature-sensitive mutants, RNA was isolated after growth at 23 and at 36°C. Most of the temperature-sensitive nonspecific ste mutations had little if any effect on STE2 mRNA levels (however, see Discussion); the threefold less RNA was found in most of the mutants at 36°C relative to 23°C, as compared to a twofold level in the wild-type a parent at 36°C (Fig. 4; Table 2). This reduction might reflect a general temperature effect on transcription of conjugation-specific genes. In contrast, the ste4-1 mutant the STE2 transcript level was at least eightfold lower at 36 than at 23°C (Fig. 4, lanes 3 and 4). This result was confirmed by analysis of poly(A)-enriched RNA isolated from other ste4 mutants, specifically from strains carrying a nonsense mutation or gene disruption in STE4 (Fig. 5). In all cases, STE2 RNA was detected at approximately 10% of the level seen in the wild-type parent. The STE4 gene product is therefore not absolutely required for STE2 transcription but is necessary for the synthesis or maintenance of normal levels of the RNA. In contrast, gene disruptions in the STE5 gene had very little effect on STE2 RNA levels (Fig. 5, lane 4), indicating that this gene product may have little or no role in STE2 expression.

Preliminary experiments suggested that STE2 transcript levels were higher in a cells that had been incubated for 2 h with α-factor (data not shown). This possibility was more directly examined by analysis of RNA samples isolated from a cells incubated with α-factor for increasing periods (Fig. 6). After only 15 min of incubation with α-factor, STE2 RNA levels increased about three- to fourfold relative to those of the ADH1 control; in this experiment, longer incubations with α-factor did not seem to lead to greater stimulation of STE2 RNA accumulation.

**Sequence of the 5' regulatory region.** Transcriptional regulation of STE2 appears to be identical to that of BARI (V. L. MacKay and T. R. Manney, manuscript in preparation), and detailed analysis of the latter gene has demonstrated that all

![FIG. 4. STE2 RNA levels in wild-type and temperature-sensitive ste mutant strains. Poly(A)-enriched RNA samples from various ste mutants and their parent strains grown at the indicated temperature were isolated, fractionated by electrophoresis, transferred to nitrocellulose, and probed with nick-translated fragments from pAH1 (either the 1.3-kb PstI-HindIII fragment or the 1.1-kb PvuII-PvuII fragment) (see Materials and Methods). After hybridization and autoradiography, the blots were cleaned and rehybridized with nick-translated pY976 which contains the ADH1 gene. (A) Lane 1, XT1177-S47c (a, 36°C); lane 2, XT1177-S47c (a, 23°C); lane 3, VZ4 (a ste4-1, 36°C); lane 4, VZ4 (a ste4-1, 23°C); lane 5, VAC1 (a ste5-1, 36°C); lane 6, VAC1 (a ste5-1, 23°C); lane 7, 381G-79A (a ste7-2, 36°C); lane 8, 381G-79A (a ste7-2, 23°C); lane 9, 381G-41A (a ste11-1, 36°C); lane 10, 381G-41A (a ste11-1, 23°C); lane 11, 381G-59C (a ste12-1, 36°C); lane 12, 381G-59C (a ste12-1, 23°C). Strain 381G, the parent of the ste7, ste11, and ste12 mutants, gave results similar to those of XT1177-S47c (data not shown).**

![FIG. 5. STE4 and STE5 influence on levels of STE2 RNA. Poly(A)-enriched RNA samples were obtained from wild-type a strains X2180-1A and G2, from derivatives of G2 in which either the chromosomal STE4 or STE5 gene has been disrupted with LEU2 or URA3 (strains kindly provided by L. Bell, ZymoGenetics), or from strains bearing nonsense mutations in the STE4 gene (see Materials and Methods). The RNA was subjected to electrophoresis, transferred, and hybridized with radiolabeled mpl1-ZY36 (the PstI-BamHI fragment from pAH1 subcloned into M13mp11). Lane 1, G2; lane 2, G2 ste4Δ; lane 3, G2; lane 4, G2 ste5Δ; lane 5, X2180-1A (a); lane 6, XL4-S47 (a ste4-4); lane 7, XL4-S118 (a ste4-4). Similar results were obtained with RNA isolated from a G2 derivative bearing a different STE4 gene disruption (data not shown).**

**TABLE 2. Quantitation of autoradiograms in Fig. 3B and 4 by scanning densitometry**  

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<th>Strain</th>
<th>STE2/ADH ratio at:</th>
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<th>23°C</th>
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<td>1.22</td>
<td>5.53</td>
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*The autoradiograms were scanned as described in Materials and Methods. The calculated areas of the STE2 and ADH bands were normalized for each autoradiogram and used to determine the STE2/ADH ratio.*
Transcriptional regulation of STE2. MATa2 regulation of genes expressed specifically in a cells was predicted in the α1-α2 hypothesis and is confirmed for the specific cases of STE6 (59), BARI (31; MacKay and Manney, in preparation), and STE2 (this work). For all of these genes, there was no detectable homologous RNA in cells that contained a functional MATa2 gene. Although MATa2 regulation of a-specific genes could occur by rapid turnover of the encoded RNAs, it is more likely that the MATa2 protein acts by blocking transcription of these genes. Recent work from other groups has identified a consensus sequence in STE6, BARI, STE2, and the α-factor structural gene MFA1 that is responsible for MATa2 repression (21, 34). In all of these genes, the consensus sequence is approximately 200 to 300 base pairs' to the translational start and probably is part of a binding site for the MATa2 protein in the nucleus (12). That the MATa2 product is sufficient for the repression at least of STE2 was indicated by the insensitivity to α-factor of mata mutant transformed with a plasmid in which the MATa2 coding sequence had been joined to the constitutive ADH1 promoter (G. Ammerer, personal communication).

Other factors also influence STE2 expression at the transcriptional level, although our data cannot discriminate between transcription initiation and RNA stability. The three- to fourfold stimulation of stable STE2 RNA levels is similar to the α-factor enhancement observed for both secreted Barrier activity (31) and BARI RNA (MacKay and Manney, in preparation) and for secreted α-factor activity (55). (It should be noted that none of these genes appears to be represented among the hormone-responsive genes described by Stettler and Thorner [52].) Similarly, the abundance of the α-specific RNA encoded by STE3 (proposed to be the α-factor receptor gene) has also recently been reported to be increased approximately fourfold by incubation of a cells with α-factor (11).

A third level of STE2 regulation involves the products of at least some of the nonspecific STE genes. Using temperature-sensitive ste mutations, we could detect the effect of the ste4 (and possibly the ste12) mutation on STE2 RNA levels, and the role of the STE4 gene product was subsequently confirmed with both a ste4 nonsense mutation and gene disruptions in STE4. Although the STE4 protein is not absolutely required for the synthesis or accumulation of STE2 RNA, its deficiency effects a 10-fold reduction in STE2 transcript levels. Relative to a wild-type control, the temperature-sensitive ste12 mutation led to a two- to threefold decrease in STE2 RNA at the restrictive temperature (Table 2); however, using tighter ste12 mutations, Fields and Herskowitz have recently shown that the STE12 gene product is also required for normal levels of STE2 RNA (10). Since gene disruptions in the STE7 and STE11 genes have likewise been shown to cause decreased levels of STE3 RNA in a cells (D. Chaleff and G. Sprague, personal communication), it is possible that a similar role for these gene products in the accumulation of STE2 RNA can be demonstrated with the use of the gene disruption strains. The slight effect of a disruption in the STE5 gene on STE2 RNA levels was somewhat surprising, since high levels of STE5 gene product can suppress the mating defect of strains carrying the temperature-sensitive ste4-1 allele used in the experiments shown in Fig. 4 (28; L. Bell, K. Nasmyth, and V. L. MacKay, manuscript in preparation). Perhaps the expression of other genes required for conjugation is affected by both the STE4 and STE5 gene products. From the data accumulated in all of these studies, it is tempting to hypothesize that the products of the nonspecific STE genes may

DISCUSSION

Although the mating type of a yeast cell segregates as a single gene, the phenotypic differences among a, a, and a/α cells indicate that selective expression of several different genes is required to establish the manifested cell type. Our results have demonstrated multiple ways in which the expression of a single gene required for conjugation is regulated.
form a transcription complex that is specifically required for high-level expression of the mating-type-specific genes. Alternatively, although mutants with defects in any one of the nonspecific STE genes (STE4, STE5, STE7, STE11, or STE12) would serve as a-factor receptor; for example, the increase of STE2 transcription during incubation of a cells with a-factor is consistent with the increase of receptor on the surface of a cells (18). Jenness et al. (D. Jenness, personal communication) also found that there was a loss of a-factor binding in temperature-sensitive ste4 mutants grown at the restrictive temperature, a result that is consistent with our interpretation that the STE4 protein is involved in STE2 RNA accumulation. The loss observed for receptor activity was, however, substantially less than the decrease in STE2 RNA levels in ste4 mutants.

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