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Identification and regulation of cell-type-specific genes required for mating in Saccharomyces cerevisiae

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Identification and regulation of cell-type-specific genes required for mating in *Saccharomyces cerevisiae*

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DEDICATION

This thesis is dedicated to the memory of my parents

Florence Mae Jensen and Ben Lee Wilson,

who put me up to this mischief;

and to my grandparents

Ann Jensen and Emil Jensen

who continue to provide love and support.

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IDENTIFICATION AND REGULATION OF CELL-TYPE-SPECIFIC GENES REQUIRED FOR MATING IN SACCHAROMYCES CEREVISIAE

by Katherine Lee Wilson

ABSTRACT

The α2 product of the α mating type locus of yeast is proposed to be a negative regulator of a set of dispersed genes concerned with specialized mating properties of a cells. This set of genes includes those genes, termed a-specific sterile genes (STE2, STE6, STE14), which are required for mating by a cells but not by α cells. To understand how α2 regulates the a-specific genes I studied a gene that was potentially regulated by α2: the STE6 gene. The STE6 gene was cloned and used as a probe for STE6 gene expression. STE6 RNA is not detectable in strains containing the wildtype α2 gene product, indicating that α2 is a negative regulator of gene expression and that it acts at the level of RNA production. I determined by deletion analysis of the STE6 promoter that DNA sequences upstream of the STE6 transcription initiation sites are required for regulation by α2, and identified a 31-basepair potential operator site for α2 repression. Furthermore I identified a DNA segment located well upstream of the region required for repression that is required for full levels of STE6 expression, and have thus separated a negative regulatory region from a potential upstream activator site (UAS) for STE6. In the course of this work I determined the DNA sequence of a 593-basepair fragment that includes the 5' terminal flanking region and presumptive N-terminal

coding region of STE6, sequenced the deletion endpoints, and located the STE6 transcript initiation sites. The suggested organization of the STE6 promoter makes testable predictions about the mechanism of α2 repression.

In addition to the regulation of STE6 I also examined STE6 function, by analysis of the phenotype of a mutant carrying an insertion mutation of the STE6 gene, the ste6::lacZ allele. Along with an a-specific defect in mating, this mutant is greatly reduced (but not completely deficient) in a-factor production, whereas other phenotypes characteristic of a cells are normal. STE6 thus appears necessary for biosynthesis of a-factor. An intensive mutant hunt for new a-specific STE genes resulted in the identification of the STE16 gene. The phenotype of MATa ste16 mutants indicates that STE16 is required for a-factor production. I have therefore identified a third a-specific STE gene, in addition to STE6 and STE14, required for a-factor biosynthesis.

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CHAPTER 1

INTRODUCTION

INTRODUCTION

The work described in the following chapters concerns two themes: repression and sterility. Repression, the inhibition of gene expression or activity, was postulated to be the basis for determining two of the three specialized cell types of the yeast Saccharomyces cerevisiae. Sterility, the inability to conjugate (mate) with cells of the opposite mating type, is the phenotypic consequence of mutations in genes that are the targets of the negative regulation. Little of the molecular basis of negative regulation in yeast was known at the time this work was begun. I have studied negative regulation of STE6, a gene required for mating proficiency of a cells, by the α2 product of MAT, the mating type locus. This introductory chapter describes genetic and molecular studies of transcriptional regulation in prokaryotes and eukaryotes, with an emphasis on negative regulation since it is the focus of my work. The variety of other means by which cells regulate expression (gene rearrangements; transcription termination, antitermination, attenuation; mRNA processing, transport, stability; translational controls) will not be discussed explicitly.

Gene expression in procaryotes is largely regulated at the level of transcription initiation (reviewed by deCROMBRUGGHE et al. 1984; GOTTESMAN 1984; RAIBAUD and SCHWARTZ 1984). DNA-binding regulatory proteins activate or repress transcription initiation by mechanisms that are beginning to be understood. Some repressor binding sites (operators) are located within the RNA polymerase binding site (promoter), in which case RNA polymerase access to the promoter is blocked; this occurs in the regulation of the P_R promoter by λ

repressor and cro (PTASHNE et al. 1980; GUSSIN et al. 1983), the regulation of SOS genes by lexA (see GOTTESMAN 1984), and the regulation of the lac P_2 promoter by the cAMP receptor protein (CRP-cAMP) (REZNIKOFF 1978; MALAN and McCLURE 1984). Operators can also be located between the promoter and the gene: in one such case bound repressor may prevent RNA polymerase from moving, since CRP-cAMP (repressor) and polymerase appear to bind DNA simultaneously upstream of the crp gene (AIBA 1983). Transcription activation, on the other hand, is mediated by protein-protein contacts between DNA-bound RNA polymerase and DNA-bound activator (HOCHSCHILD et al. 1983). Activators can increase either the affinity of RNA polymerase for the promoter or the rate of isomerization of bound RNA polymerase from the transcriptionally inert "closed complex" to the active "open complex" (CHAMBERLAIN 1974; McCLURE 1980; HAWLEY and McCLURE 1982). In some cases activation is also achieved indirectly by the occupation of competing or overlapping promoters (MUSSO et al. 1977; MALAN and McCLURE 1984). However no positive control mechanisms are yet proven. Many procaryotic regulatory proteins (for example, λ repressor and cro [PTASHNE et al. 1980], CRP-cAMP [deCROMBRUGGHE et al. 1984], and AraC [HAHN et al. 1985; LEE et al. 1981]) can function as repressors, activators, or both simultaneously, depending upon the particular locus and site(s) bound. Proteins belonging to a special class of activators act as sigma subunits of RNA polymerase and alter promoter selection to specific sets of genes such as heat shock response genes in E coli. (GROSSMAN et al. 1984) and developmentally regulated genes in B. subtilis (LOSICK and PERO 1981; JOHNSON et al. 1983).

There are interesting exceptions to the two-dimensional cases of procaryotic regulation described above, that indicate longer range interactions between regulatory proteins reminiscent of eukaryotic gene regulation (discussed below). Physical interaction between two separate operators was originally proposed to explain repression of the gal operon by two gal repressor binding sites located approximately 110 basepairs apart, one of which lies within the galE coding sequence (IRANI et al. 1983). Single mutations at either operator cause constitutive, repressor-insensitive expression of the operon, indicating that occupation of both sites is required for repression of transcription. Similarly, full repression of the araBAD operon under noninducing conditions involves an interaction between two AraC protein binding sites. The araI site lies immediately upstream of the araBAD promoter, while the $araO_2$ site is located over 200 basepairs upstream of araI. An elevated basal level of araBAD gene transcription is observed in strains deleted for the $araO_2$ site (DUNN et al. 1984). AraC protein dimers bound to the two AraC binding sites are proposed to interact via a loop in the intervening DNA, based on experiments that altered the distance between the two operator sites: full repression of the operon was still observed when the distance was varied by integral helix turn lengths, but not when the spacing between the two operators was altered by fractions of helix turn lengths (HENDRICKSON et al. 1983; HAHN et al.1985).

In contrast to most bacterial operons, the regulation of eukaryotic transcription initation by RNA polymerase II is mediated through sites located far away from the sites where transcription

initiates (BREATHNACH and CHAMBON 1981; McKNIGHT 1982). In higher eukaryotes these sites are called enhancers (reviewed in BENOIST and CHAMBON 1981; GLUZMAN and SHENK 1983; KHOURY and GRUSS 1983). Enhancers stimulate the efficiency of transcription initiation at promoters fused in *cis*. Enhancers work in both orientations with respect to transcription start sites and over a wide range of distances, but proximal promoters are activated in preference to more distal ones (WASYLYK *et al*. 1983). Some enhancers exert their effects when located thousands of basepairs from the transcription start or when downstream of the coding sequence of a gene.

The function of a given enhancer has often been found to be dependent upon specific physiological or developmental conditions, indicating that specific enhancers may function only in response to a particular signal or set of signals (SCHOLER and GRUSS 1984). For instance, enhancer elements found near immunoglobulin genes are active only in lymphocyte-derived cells at a particular stage of differentiation and depend for activity upon factors expressed only in that particular cell type (BANERJI *et al*. 1983; GILLIES *et al*. 1983; QUEEN and BALTIMORE 1983). The best evidence for interactions between enhancers and specific regulatory proteins comes from studies of the glucocorticoid response of the mouse mammary tumor virus (MTV) in the genome of mouse cell lines (reviewed in YAMAMOTO 1984). Yamamoto and colleagues have shown that transcription activation *in vivo* is dependent upon binding of the glucocorticoid recepter to sites called glucocorticoid response elements (GRE) in the presence of the synthetic glucocorticoid dexamethasone.

Alterations in chromatin structure have been invoked as one means by which regulatory proteins could stimulate changes in gene transcription. It is not known how the binding of the receptor to the GRE stimulates transcription, but a plausible mechanism is that the receptor-enhancer interaction provides a bidirectional RNA polymerase entry site, perhaps by altering the chromatin structure of the region. There is experimental evidence that receptor binding induces chromatin structure alterations that persist even after hormone withdrawal, when transcription ceases (ZARET and YAMAMOTO 1984; reviewed in BROWN 1984 and YAMAMOTO 1985). Eukaryotic genes in general are thought to be transcriptionally inactive in most cell types because of "repressive" chromatin structure which is altered for particular genes by the action of specific activator proteins. To explain the extreme repression of nonexpressed, tissue-specific genes, WEINTRAUB (1984) has proposed that histone H1 proteins stabilize nucleosomes in a crosslinked state that makes genes refractory to basal expression or low-level induction.

Eukaryotic regulatory proteins like procaryotic regulators may be capable of mediating either positive or negative regulation. There is preliminary evidence that glucocorticoid receptors may also mediate repression; treatment of newborn rats with dexamethasone turns off transcription of the α-fetoprotein (αFP) gene in liver cells (GUERTIN et al. 1983). The kinetics of transcriptional repression are consistent with it being a direct effect of glucocorticoid receptor binding to the αFP gene, but this has not yet been proven. Inhibitive effects of dexamethasone on rDNA transcription have been attributed to inhibition of a protein factor required for transcription by RNA

polymerase I (CAVANOUGH et al. 1984). If the observation of αFP repression is shown to be a direct consequence of receptor binding, then proposed mechanisms for enhancer function must account for both possible outcomes (activation or repression), at least for enhancers recognized by the glucocorticoid receptor.

There is a well-characterized example of transcription repression in a eukaryotic virus, SV40, where early gene transcription is repressed by the binding of tetramers of the repressor (T antigen) to three sites located at and around the transcription initiation site (BENOIST and CHAMBON 1981; GRUSS et al. 1981; DYNAN and TJIAN 1983; RIO and TJIAN 1983; JONES and TJIAN 1984). Binding to sites I and II blocks transcription initiation but does not block transcription elongation when the sites are located downstream of the initiation sites of heterologous promoters (MYERS et al 1981). T-antigen thus appears to repress transcription initiation by simple "procaryotic" means: repressor binding prevents stable polymerase binding. Interestingly, the mammalian HMG CoA reductase gene is subject to end-product repression at the transcriptional level like SV40 early gene transcription, and its promoter resembles the SV40 promoter (REYNOLDS et al. 1984).

Enhancer-like elements known as upstream activation sites (UASs) have been described in yeast (reviewed by GUARENTE 1984). A UAS is defined as a DNA fragment that is sufficient to activate transcription of heterologous genes to which it is fused in cis, in response to the specific physiological signals that stimulate transcription of the

gene from which the UAS was originally derived (BEIER and YOUNG 1982; GUARENTE et al. 1982; GUARENTE and MASON 1983). A yeast UAS is simultaneously a promoter element and a regulatory element. UASs, like enhancer sequences, still function when moved hundreds of basepairs from their native positions and when positioned in either orientation upstream of the transcription start (GUARENTE and HOAR 1984). Unlike enhancers, however, no yeast UAS has yet been found to function from a downstream position with respect to the transcription start (GUARENTE and HOAR 1984). Detailed analysis has identified UASs upstream of CYC1 (GUARENTE and MASON 1983; GUARENTE et al. 1984), GAL10-GAL1 (GUARENTE et al. 1982; JOHNSTON and DAVIS 1984; WEST et al. 1984), ADR1 (BEIER and YOUNG 1982), and HIS3 and HIS4 (STRUHL 1982; DONAHUE et al. 1983; HINNEBUSCH and FINK 1983a). Candidates for the regulators that interact with UAS sequences have been identified by genetic analysis (DENIS et al. 1981; OSHIMA 1982; HINNEBUSCH and FINK 1983b; DENIS 1984; GUARENTE et al 1984; HINNEBUSCH 1984). For example, mutants defective in GAL4 function fail to express genes required for galactose metabolism (OSHIMA 1982; St. JOHN and DAVIS 1979).

The existence of negative regulation in yeast was demonstrated genetically, with the discovery of recessive mutations that caused constitutive expression of genes unlinked to the mutation. For example, yeast normally do not express genes for the metabolism of galactose in the absence of inducer (galactose), but strains carrying recessive mutations in the GAL80 gene do express galactose metabolic functions (OSHIMA 1982). However, the GAL80 gene product is thought to inhibit expression of the galactose cluster genes through inhibition of

the activator protein GAL4, because constitutive expression in gal80 strains still depends upon GAL4 and because $GAL4^{c}$ alleles have been isolated that are insensitive to inhibition by the GAL80 gene product and constitutively activate high levels of expression of galactose metabolic genes.

There are fewer examples of negative regulation at the level of transcription in yeast. Carbon catabolite repression (by glucose) has been observed for the transcription of ADR2 (DENIS et al. 1983), CYC1 (GUARENTE et al. 1984), and GAL (ADAMS 1972; MATSUMOTO et al. 1983) and shown by deletion analysis to be mediated through DNA sequences upstream of the transcript start sites. It is not clear, however, whether glucose repression is mediated through negative effects on positive regulators (see MATSUMOTO et al. 1983; DENIS 1984; NEIGEBORN and CARLSON 1984), or through direct negative interactions at DNA regulatory sites. The 365-basepair GAL10-GAL1 regulatory region, including sequences responsible for glucose repression, confer glucose-repressibility when placed upstream of an intact HIS3 promoter (STRUHL 1984; STRUHL 1985). However, repression was observed when the GAL site was located 10-80 basepairs but not 130 basepairs upstream of the HIS3 UAS. In different experiments, deletions between the GAL UAS and the GAL1 TATA box reduced glucose repression from ca. 150-fold to ca. 5-10-fold, and residual expression was not GAL4-dependent, indicating that glucose repression is not (wholly) mediated through effects on GAL4 activity (WEST et al. 1984). (The GAL80 protein does not mediate carbon catabolite repression [TORCHIA et al. 1984].)

The second example of negative regulation in yeast concerns the silent copies of the mating type locus located at HML and HMR (reviewed in HERSKOWITZ and OSHIMA 1981). The action of six different SIR gene products (KASSIR and SIMCHEN 1985; RINE and HERSKOWITZ 1985; G. SIMCHEN, pers. comm.) is required to prevent transcription of HML and HMR (RINE et al. 1979; KLAR et al. 1981; NASMYTH et al. 1981) and to prevent the silent copies from serving as recipients in HO-mediated transposition events (KOSTRIKAN et al. 1983; KLAR et al. 1981; NASMYTH et al. 1981). Repression by SIR depends upon DNA sequences located in cis to HML and HMR, 700 to 1400 basepairs away from the sites of potential transcription initiation at each silent locus (ABRAHAM et al. 1984; FELDMAN et al. 1984). Sir can regulate heterologous (non-mating-type related) promoters substituted for mating type information at HMR. The mechanism of SIR repression is unknown, but a 270-basepair site (HMRE) through which SIR repression acts has enhancer-like qualities: it is orientation-independent and functions both 5' and 3' to HMR; in addition repression can extend through one MATa1 promoter to a second MATa1 promoter 2700 basepairs distant (BRAND et al. 1985). Different SIR proteins may interact to form a complex or structure; many pairwise combinations of nonallelic, recessive mutations fail to complement in doubly heterozygous diploids (KASSIR and SIMCHEN 1985; RINE and HERSKOWITZ 1985).

Curiously, some yeast regulatory regions have autonomous replicating activity ("ARS" sites; BROACH et al.): the HMRE site of SIR repression has ARS activity, and MILLER and NASMYTH (1984) have provided evidence that replication is required to establish SIR

repression over HMR but not to derepress the locus by a shift to high temperature in a sir^{ts} strain.

Genetic analysis of the regulation of yeast cell type by the mating type locus (MAT) suggested the existence of two negative regulatory activities called α2 and a1-α2, and one positive regulator called α1 (MacKAY and MANNEY 1974a,b; STRATHERN et al. 1981). Each regulator was proposed on genetic grounds to determine the expression or activity of the family of genes required to establish one of the three cell types-- the a or α haploid cell type or the a/α diploid cell type. At the time my work began, two of these regulators (α1 and a1-α2) had been shown to determine the expression of cell-type specific genes at the level of transcription. First, the α1 activity had been shown by SPRAGUE et al (1983) to activate transcription of the STE3 gene: stable STE3 RNA was not detectable in strains lacking α1 activity (such as MATa strains and matα1 mutants). Second, the a1-α2 activity had been shown to inhibit stable RNA production from MATα1 (KLAR et al. 1981; NASMYTH et al. 1981; STRATHERN et al. 1981) and HO (JENSEN et al. 1983): RNA from these genes was not detectable in any strain producing both wildtype α2 and a1 proteins. (Transcription of the repeated elements Ty1 is greatly reduced by the action of α2 and a1 [ELDER et al. 1981]). It was not known at what level α2 regulated genes proposed to be inhibited by it (SPRAGUE and HERSKOWITZ 1981).

The regulatory protein α2 was proposed on genetic grounds to play an important role in establishing yeast cell type. First, α2 was proposed to be a negative regulator of genes required for cells to mate

as a (the "a-specific" genes) because mutants carrying recessive α2 mutations constitutively produced a-specific gene products. Second, in the presence of the a1 protein, α2 acquired a second specificity for the inhibition of the family of genes turned off in MATa/MATα diploid cells. How did the α2 protein work? Based on the genetic prediction that α2 was a negative regulator and the precedent that the other two mating type regulators acted at the level of transcription (RNA accumulation), I tested the hypothesis that α2 inhibits a-specific gene expression at the level of transcription. However, there was no promise that any of the mating type regulatory proteins were direct regulators of transcription. As described above there are negative regulators such as GAL80 that may act by inhibiting activators. Another possible mechanism for negative regulation is that the proposed negative regulators function by activating repressors.

To understand the mechanism of α2 inhibition, namely the regulation of the a-specific genes by α2, I studied a gene that was potentially regulated by α2: the STE6 gene. The STE6 gene was cloned and used to determine that STE6 is indeed regulated at the level of transcription by α2 (Chapter 2; WILSON and HERSKOWITZ 1984). I determined by deletion analysis of the STE6 promoter that DNA sequences upstream of the STE6 transcription initiation sites are required for regulation by α2, and identified a 31-basepair potential operator site for α2 repression (Chapter 3). Furthermore I identified a potential UAS that is separate from and upstream of the site of α2 repression. The proposed organization of the STE6 promoter makes testable predictions for the mechanism of α2 repression.

In addition to studying the regulation of the STE6 gene, I studied its function and determined by the phenotypic analysis of a presumptive ste6 null mutant that STE6 is required for the biosynthesis of a-factor, the peptide mating pheromone of a cells (Chapter 2). I conducted a systematic mutant hunt for a-specific STE genes (those required for mating by a cells but not by α cells), and isolated all the known a-specific STE genes as well as a new a-specific STE gene called STE16 (Chapter 4). I show that STE16 is also required for a-factor biosynthesis. The identification of STE16 (Chapter 4) and the identification of a temperature-sensitive allele of STE6 (Appendix 1) are most relevent for the biochemical investigation of mating physiology: STE16, STE6, and STE14 are all three required for the processing and/or secretion of a-factor.

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CHAPTER 2

NEGATIVE REGULATION OF *STE6* GENE EXPRESSION BY THE α2 PRODUCT OF *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

The α2 product of the α mating type locus of yeast is proposed to be a negative regulator of a set of dispersed genes concerned with specialized properties of *a* cells. This set of genes includes those genes, termed *a*-specific *STE* genes (*STE2*, *STE6*, and *STE14*), which are required for mating by *a* cells but not by α cells. We have cloned the *STE6* gene to determine whether its expression is limited to *a* cells and, if so, whether its expression is inhibited in α cells by the α2 product. Expression of *STE6* has been assayed in two ways: by blot hybridization of RNA and by B-galactosidase activity in strains carrying a *STE6-lacZ* hybrid gene. We find that *STE6* expression is limited to *a* cells and is negatively regulated by the α2 product. *STE6* RNA is not detectable in strains containing the wildtype α2 gene product. Expression of *STE6* is at least 150-fold lower in α cells than in *a* cells, based on B-galactosidase activities in *a* and α cells carrying the *STE6-lacZ* gene. These results confirm that the α2 product is a negative regulator of gene expression and show that it acts at the level of RNA production. We have also examined the phenotype of a mutant carrying an insertion mutation of the *STE6* gene, the *ste6::lacZ* allele. In addition to an *a*-specific defect in mating, this mutant is greatly reduced (but not completely deficient) in *a*-factor production. Other phenotypes characteristic of *a* cells-- Barrier activity, agglutination, and response to α-factor-- are normal. *STE6* thus appears necessary for biosynthesis of *a*-factor.

INTRODUCTION

The three cell types of yeast (*a*, α, and *a*/α) exhibit many differences in phenotype, though they differ genetically at only a single locus, *MAT*, the mating type locus. For example, *a* and α cells each secrete an oligopeptide pheromone, called *a*-factor and α-factor respectively, and respond to the pheromone of the opposite type (10, reviewed in reference 36). In addition *a* cells degrade α-factor in a process requiring the *BAR1* gene (17, 34). In contrast, *a*/α diploids neither produce nor respond to sex pheromones but are instead capable of undergoing meiosis and sporulation under the appropriate conditions. α cells carry the *MAT*α allele, *a* cells the *MAT*a allele, and *a*/α cells (formed by mating between *a* and α cells) contain both *MAT* alleles.

MacKay and Manney proposed that *MAT* is a regulatory locus controlling the expression of genes unlinked to it, based on the finding that mutations affecting mating ability map to loci distinct from *MAT* (24, 25). Some genes, such as *STE5*, are required for mating of both *a* and α cells. Others have a phenotype in only one cell type. Mutations at three loci--*STE2* (24, 25), *STE6* (34), and *STE14* (L. C. Blair, Ph.D. thesis, University of Oregon, Eugene, 1979)--affect mating ability only in *MAT*a strains; these genes are termed "*a*-specific" *STE* genes. Similarly, mutations at four loci--*STE3* (24, 25), *STE13* (35), *KEX2* (23), and *TUP1* (42)--affect mating only by *MAT*α cells and define a set of α-specific *STE* genes. It is plausible to propose that expression of *a*- or α-specific genes may be limited to their respective cell type. The *a*- and α-specific *STE* genes represent some but not all of the genes whose expression might be limited to one cell type; for

instance, the Barrier phenotype is exhibited only by *a* cells and not in the other cell types.

Strathern *et al* (38) proposed a specific model for control of cell type in which the mating type locus alleles code for three regulatory activities: α1, α2, and *a*1-α2 (see Figure 1). *MAT*α codes for two proteins, α1 and α2: α1 is a *positive* regulator of at least some genes that are required for the α cell type; α2 is a *negative* regulator of genes that are required for the *a* cell type. In *MAT*a cells the *a*-specific genes are expressed constitutively because there is no α2 protein; α-specific genes are silent due to the absence of α1 protein. *a*/α cells contain the third regulatory activity, *a*1-α2, which requires α2 from *MAT*α and the *a*1 gene product of *MAT*a. Mating is turned off in *a*/α cells because α2 inhibits expression of *a*-specific genes and because *a*1-α2 turns off production of α1. α2 thus has a dual role, by itself as the negative regulator of the *a*-specific family, and together with the *a*1 protein as the negative regulator of genes turned off in *a*/α diploids.

At what level do the regulatory activities encoded by *MAT* act? α1 and *a*1-α2 have been shown to regulate RNA synthesis. Production of RNA from the α-specific *STE3* gene is dependent upon α1 protein (37); similar results have been obtained for *MF*α*1*, the major structural gene for α-factor (R. Jensen, K. Wilson, and S. Fields, unpublished observations). RNA production from *HO* (18), *MAT*α*1* (20, 29) and *STE5* (V. MacKay, J. Thorner, and K. Nasmyth, personal communication) have been shown to be negatively regulated by *a*1-α2. We note that

expression of STE genes is not necessarily limited to one or another cell type: the α-specific STE13 gene is expressed in all yeast cell types (G. Sprague, Jr. and R. Jensen, unpublished observations, cited in 37). TUP1 and KEX2, despite being α-specific genes with respect to mating phenotype, are not limited in expression to one cell type because mutations at these loci exhibit phenotypes (unrelated to mating) in all three cell types (23, 42).

The focus of this paper is the regulation of an a-specific gene by the α2 product of MATα. The proposal that α2 is a negative regulator of a-specific genes comes from analysis of yeast mutants defective in the MATα2 gene. In particular, matα2$^-$ mutants exhibit several properties characteristic of a cells: they mate (inefficiently) with α cells, they exhibit the a-cell-specific Bar function, and they produce a-factor (38; reviewed in 16). Since the matα2 mutations tested are recessive to MATα2, it was proposed that α2 is a negative regulator of functions specific to a cells. Subsequent genetic and physiological analyses (21, 34, 38) support this view.

We ask here whether expression of the a-specific gene STE6 is limited to a cells and whether its expression is turned off by the α2 product. We describe the cloning of STE6 and its use in addressing these questions. We find that production of stable STE6 mRNA is limited to a cells and is under negative regulation by α2. In addition, we have constructed an insertion mutation of STE6 and studied its phenotype to assess the physiological role of STE6 in mating.

MATERIALS AND METHODS

Strains, plasmids and media. Yeast strains used are listed in Table 1. Bacterial strains were DB6507 (thr leuB pro recA pyrF::Tn5 hsdR hsdM; obtained from D. Botstein) and MC1066 (leuB600 trpC9830 pyrF::Tn5 lacX74 rpsL hsdR galU galK; obtained from M. Casadaban via M. Hall). Yeast strains HR125-5d and 1369, constructed by mating type interconversion from HR125-5d, were kindly provided by Rob Jensen.

Plasmids used were pBR322 (3), YEp13 (pBR322 containing the yeast LEU2 gene and portions of 2u DNA (4)), YIp5 (pBR322 containing the yeast URA3 gene (40)), YCp50 (YIp5 carrying yeast centromere CEN4 and an ARS, obtained from C. Mann), and pMC1871 (pBR322 containing a lacZ gene flanked by polylinkers, obtained from M. Casadaban (5)).

The ste6::lacZ fusion was constructed as follows (see Figure 2). The 5.5 kilobasepair (kbp) yeast DNA fragment of plasmid YEpSTE6 was transferred into pBR322 via the vector HindIII and SphI sites. The resultant plasmid pBR322-STE6 was partially digested with PstI, ligated with PstI-cut plasmid pMC1871 (containing the lacZ gene) and transformed into E. coli strain MC1066. Transformants were selected by ampicillin resistance and screened for B-galactosidase activity by including XG in the plates. Plasmid DNA was isolated from blue Ap^r colonies and screened by restriction analysis. Of six candidates, all contained the 3 kbp lacZ PstI fragment inserted as shown in Figure 2. One of these candidates, designated pBR322-ste6::lacZ, was used for the gene replacement analysis.

Yeast media were prepared as described (17). Media containing 5-bromo-4-chloro-3-indolyl-™-D-galactoside (XG; Boehringer Mannheim Biochemicals) was prepared as described (30) except that yeast XG

plates contained approximately 0.7 mg/ml XG.

Genetic manipulations. Genetic manipulations were performed as described (34). Matings involving mating-deficient strains were performed by selection for prototrophy or were facilitated by including the YEpSTE6 plasmid (containing the STE6 gene) in the ste6 strain. In the latter case further analysis was performed on diploids that had lost the plasmid, obtained by screening for cells that became Leu^- after growth on rich medium.

Isolation of yeast DNA fragment carrying the STE6 gene. A yeast clone bank constructed in vector YEp13 (4) by Sau3A partial digestion of DNA from yeast strain AB320 (28) was kindly provided in E. coli by K. Nasmyth. Plasmid DNA was isolated and used to transform ste6 strain K39-3b by the method of Beggs (1). Yeast transformants were selected as leucine prototrophs on synthetic medium lacking leucine, then collected, replated and tested for ability to mate as a by replica-plating with α tester strain 70. Strain K39-3b is somewhat temperature sensitive for mating and gives rise to numerous prototrophic colonies on a patch test for mating at 30°. To facilitate screening transformant colonies, mating was performed at 32.5°, which reduces the background of mating. Plasmid DNA was isolated as described (28) from the one colony that exhibited mating and used to transform E. coli strains by standard methods.

Preparation and hybridization of yeast RNA. Yeast RNA was isolated as described by Jensen et al (18). $Poly(A)^+$ RNA was isolated using

poly(U)-Sepharose-4B and fractionated on 1.5% agarose/6% formaldehyde (9), except that Hepes buffer (pH 7.8) was used instead of borate buffer. RNA was transferred to nitrocellulose (Sartorius) and hybridized with probe as in reference 41, except that dextran sulfate was omitted from the hybridization solutions. Probe was prepared by nick-translation of plasmid YIp5-STE6 DNA using a New England Nuclear nick-translation kit. Washed filters were autoradiographed for 48-72 hours with Kodak XAR-2 film and a DuPont 1 Lightning Plus intensifying screen. Sizes of the RNA species were estimated by comparison of the autoradiograph with the rRNA bands in an ethidium bromide-stained lane of the original gel. Autoradiographs were scanned using a Zeineh Soft Laser scanning densitometer; peak areas were determined by cutting out and weighing.

Gene replacement with ste6::lacZ. The STE6 gene of HR125-5d was replaced with the ste6::lacZ allele by the method of Rothstein (31) with the following modifications. To generate a linear fragment containing yeast sequences at both ends, we digested plasmid pBR322-ste6::lacZ at the unique HindIII site in the vector (see Figure 2), removed approximately 1000 basepairs from the end by BAL31 exonuclease digestion to leave yeast sequences at that end, made a second cut at the unique KpnI site in the yeast DNA and used the entire mixture to transform strain HR125-5d. The linear fragments were cotransformed with uncut plasmid YEp13 containing the selectable LEU2 marker, and Leu^+ transformants were screened as described in the text.

Assays of mating phenotype. Mating type was determined as in reference 34 by replica-plating patches of the strains to be tested (which are auxotrophic) onto SD minimal agar plates previously spread with lawns (in rich broth) of either a (strain 227) or α (strain 70) testers containing complementary auxotrophic mutations. Diploids are prototrophic and grow on minimal medium. Mating type a was indicated by the formation of prototrophs after mating with the α lawn, and mating type α was indicated by formation of prototrophs with the a lawn.

a-factor production was assayed by replica-plating strains to YEPD plates previously spread with a thin lawn (1-5 x 10^5 cells per plate; L. C. Blair, personal communication) of strain RC757 (6, 7) followed by incubation at room temperature (22^{o}-25^{o}). a-factor production was judged by halo size relative to a standard a strain (either HR125-5d or 227) on the same plate. α-factor production was tested by bioassay on plates at room temperature as described (34) using strain XMB4-12b.

α-factor response of single cells (17) and the plate assay for Barrier activity (34) were performed as described, using strain 70 as the source of α-factor.

Agglutination assays were performed in rich medium as described by Sprague et al (35) by mixing exponentially growing cultures together and visually monitoring the production of large aggregates over the ensuing two hours. The strains tested were the HR125-5d isogenic series: a, α (strain 1369) and a ste6::lacZ (strain K77), in every pairwise combination. Quantitative efficiency of mating was determined as in reference 34. Known numbers of cells of the strains being tested were spread on minimal medium agar plates with 3 x 10^7 cells of mating

tester strain XT1177-S245C and incubated at 30°. 4 x 10^7 cells of each strain were also spread alone on minimal plates to test for reversion of auxotrophic markers: no prototrophs were observed. Efficiency of mating was calculated as the ratio of the titer of cells able to mate to the titer of total cells as assayed on YEPD plates.

B-galactosidase assays. B-galactosidase assays were performed as described in Miller (27) using chloroform/SDS to permeabilize the cells. For each segregant shown in Table 2 one culture was grown and divided into 5 aliquots; B-galactosidase activity was assayed over a three hour period with each aliquot being one time point.

RESULTS

Isolation of a cloned DNA segment which complements the mating defect of a ste6 mutant. We have isolated a plasmid that carries STE6 by screening for plasmids that allow MATa ste6 strains to mate. A MATa ste6 leu2 strain (K39-3b) was transformed with a plasmid pool comprised of random Sau3A genomic yeast DNA fragments inserted into the LEU2$^+$ vector YEp13. 18,000 independent Leu$^+$ transformants were collected, replated on medium lacking leucine, and screened for ability to mate as a as described in Materials and Methods. One such colony was obtained. It contained a plasmid (YEpSTE6) which allowed the recipient ste6 cells to mate with α tester strain 70. The restriction map of the 5.5 kbp yeast DNA insert of YEpSTE6 is shown in Figure 2.

YEpSTE6 carries the STE6 gene. To determine whether the 5.5 kbp insert in YEpSTE6 carries the STE6 gene and not a different gene with overlapping function, we allowed a plasmid containing the putative STE6 DNA to recombine with homologous sequences in the chromosome and mapped the site of integration. If the cloned DNA segment contains STE6, then the plasmid should integrate at the STE6 locus. For this analysis, the entire 5.5 kbp insert of YEpSTE6 was subcloned into the URA3$^+$ plasmid YIp5, and the resultant plasmid (YIp5-STE6) was introduced into MATa STE6 ura3-52 cells (strain HR125-5d) by transformation. Stable Ura$^+$ transformants result from integration of the plasmid into the genome; integration at the ura3 locus is greatly reduced by using the ura3-52 mutation in the recipient strain (32). Transformants with a stable Ura$^+$ phenotype were crossed to MATα ste6 ura3 strain K72-33a. The resultant diploids were sporulated, and the haploid meiotic products

were analyzed for mating phenotype and Ura phenotype. In crosses with 2 independently isolated Ura^+ integrants (K73-1 and K73-2), the Ura^+ phenotype contributed by YIp5-STE6 was tightly linked to the STE6 locus: no recombinants were observed in 65 tetrads--all MATa spores were either mating-proficient and Ura^+ or mating-deficient and Ura^-, like the parents. These results show that URA3 is now located at the STE6 locus and therefore that the 5.5 kbp segment contains nucleotide sequences present at this locus.

Even though YIp5 is incapable of autonomous replication (39), plasmid YIp5-STE6 yielded unstable transformants with high efficiency, indicating that the yeast insert may contain a sequence (an ARS sequence (38)) that allows autonomous replication of YIp5.

One-step gene replacement creates an insertion mutation in STE6. We used the one-step gene replacement scheme of Rothstein (31) to replace the wildtype STE6 gene with a ste6::lacZ allele. The ste6::lacZ mutation was constructed by inserting a 3 kbp PstI-PstI fragment containing lacZ into a PstI site of the cloned DNA (see Figure 2). The lacZ fragment contains the entire lacZ coding sequence beginning at the eighth amino acid (5). Thus, expression of this lacZ segment in yeast is dependent upon an external promoter and an in-frame translation initiation codon. The ste6::lacZ fusion was constructed for the following reasons. First, we anticipated that the lacZ insertion would disrupt the normal STE6 gene to create a null mutation at STE6. Second, determination of the chromosomal locus where the ste6::lacZ allele is introduced would confirm that the cloned DNA indeed contains

STE6 sequences. Finally, we could infer the orientation of the STE6 gene on the cloned DNA if an active fusion protein were made (12).

The ste6::lacZ DNA fragment, linearized as described in Materials and Methods, was mixed with uncut YEp13 in a molar ratio of approximately 100:1 (fragment:YEp13) and transformed into a mating-proficient MATa STE6 leu2 yeast recipient (HR125-5d). The $LEU2^+$ plasmid YEp13 was included as a selectable marker for cells that successfully took up DNA. Leu^+ transformants were replated for single colonies and screened for mating ability, since we anticipated that cells that had undergone one-step gene replacement with the ste6::lacZ DNA might show a $Ste6^-$ phenotype. This was indeed the case: 0.5% of the transformants had become mating-deficient, and all of these transformants contained the lacZ insert. The presence of the lacZ insert was scored by its enzymatic activity (blue colonies on XG indicator plates).

We have analyzed one such mating-deficient transformant (strain K77) by the method of Southern (33) using as a hybridization probe the plasmid pBR322-ste6::lacZ (data not shown). The data are consistent with a simple substitution of STE6 by ste6::lacZ.

Confirmation that the lacZ insertion allele is located at the STE6 locus was obtained in the following way: we analyzed the meiotic progeny of a cross (K59) between the sterile transformant K77 (MATa ste6::lacZ) and an α strain (K49-4b) carrying a ste6 mutation. All MATa segregants obtained in 37 tetrads were unable to mate, indicating

tight linkage between the ste6 locus and the ste mutation contributed by the transformant. In addition, half of the MATa segregants (which must carry the ste6::lacZ allele) formed blue colonies on XG indicator plates.

The ste mutation caused by the lacZ insertion is a mutation of the STE6 gene, because the ste6::lacZ allele, carried on the low copy number plasmid YCp50, fails to complement the mating defect of strain K69-1d (MATa ste6-21). Similarly, the ste6::lacZ allele at its chromosomal position fails to complement the ste6-21 mutation in MATa/MATa diploid cells. (Control experiments show that the ste6::lacZ mutation is recessive to STE6). We note that insertion of lacZ into the BamHI site located approximately 200 bp from the PstI insertion site also renders the original YEpSTE6 plasmid unable to complement a ste6 mutation.

Phenotype of the MATa ste6::lacZ mutant. Three previously-isolated ste6 mutants and eight new ste6 mutants isolated in our laboratory (K. Wilson, unpublished observations) are all leaky in phenotype: the ability to mate is greatly decreased but not completely abolished. The mating efficiency of strain K39-3b, for instance, is 3×10^{-3} at room temperature. It was therefore of interest to determine the phenotype of a null mutation, with the expectation that a 3 kbp insertion into STE6 would severely inactivate this gene. The ste6::lacZ mutant manifests severe loss of mating ability: its mating efficiency is 5×10^{-7}. The wildtype parent mates with an efficiency of 0.86 (assays as in Materials and Methods). Thus the ste6::lacZ mutation affects mating

much more than previously existing mutants.

a strains carrying the ste6::lacZ mutation are defective in the production of the pheromone a-factor. The a-factor deficiency cosegregates with the ste6::lacZ allele in all 39 tetrads from diploid K59. a-factor production from an isogenic set of strains--mutant MATa ste6::lacZ and its MATa STE6 parent and a MATα control--is shown in Figure 3. The a-factor halo produced by the ste6 mutant is greatly reduced, but a small halo is visible. a-factor production is the only a-specific function that we have tested that is abnormal in the ste6::lacZ mutant. The mutant cells are normal in their ability to respond to α-factor by cell cycle arrest (14) and morphological change ("shmoo") as shown previously (J. D. Rine, Ph.D. thesis, University of Oregon, Eugene, 1979); furthermore, the mutant exhibits Barrier activity, and mutant and wildtype a cells agglutinate with α cells equally well by one hour after mixing.

Control of STE6 RNA synthesis by the mating type locus. To determine whether the mating type locus controls expression of the STE6 gene, we used the cloned STE6 gene to assay RNA production from this gene in the three cell types, a, α, and a/α, and in the two mutants matα1 and matα2. The strains, isogenic except at the mating type locus, are derivatives of strain XT1177-S245C.

Poly(A)$^+$ RNA, size fractionated as described in Materials and Methods, was hybridized with a probe (YIp5-STE6) that contains URA3 and STE6. As shown in Figure 4, we detect three species of RNA

complementary to the probe in MATa cells. The smallest (0.9-1.0 kb) corresponds to the URA3 transcript (M. Rose, personal communication) and is present at similar levels in all strains. A second species (band x), approximately 3 kb in length, is also present in all strains. The largest transcript, approximately 4 kb in length, is present in MATa cells but is undetectable in MATα, MATa/MATα, or matα1 cells. However, the 4 kb transcript is present in cells carrying the matα2 mutation (including a matα2/MATa diploid, shown in Figure 5). In other words, the 4 kb RNA is absent from strains that contain the wildtype α2 gene product (MATα and matα1 haploids and MATa/MATα diploids).

The 4 kb RNA species appears to be the STE6 transcript. We have analyzed poly(A)$^+$ RNA from the ste6::lacZ strain and its isogenic STE6 parent and find that the 4 kb RNA species is absent in the ste6::lacZ strain, replaced by one larger and two smaller RNA species (Figure 5). The alteration in the 4 kb transcript as a result of the lacZ insertion mutation indicates that this species is the STE6 transcript. Why the lacZ insertion into STE6 results in three poly(A)$^+$ RNA species is not known. In contrast, the 3 kb band is unaltered in the ste6::lacZ strain and thus appears to be unrelated to STE6.

These results indicate that the STE6 transcript is cell-type-specific and that transcription of the STE6 gene is under negative regulation by the MATα2 gene product. We cannot determine from this analysis whether the action of α2 blocks transcription of STE6 or affects STE6 RNA stability. The abundance of the STE6 transcript in a cells, estimated from densitometric scans of the Northern

autoradiograph, is approximately the same as that of the URA3 transcript (approximately 3 copies per cell (22)). STE6 RNA is undetectable in α cells. The level of STE6 RNA in $\alpha 2^-$ strains (normalized to URA3 RNA) is at least 1.5 times greater than the level in a cells. The reason for the difference between $\alpha 2^-$ and a cells is under study.

B-galactosidase activity in MATa ste6::lacZ strains is under MAT control. To quantitate the difference in STE6 expression between different cell types and to determine if B-galactosidase expression from the chromosomal ste6::lacZ fusion gene is controlled by MAT, we analyzed segregants from diploid K61, which was formed by crossing the insertion mutant K77 (MATa ste6::lacZ) with strain 1369 (MATα STE6). Segregants from K61, which comprise an isogenic set of strains that differ only at MAT and STE6, were analyzed for mating phenotype and B-galactosidase activity. Since only half of the spores carry ste6::lacZ, the half that carry STE6 constitute the negative controls for B-galactosidase activity. Results from two complete tetrads are shown in Table 2. In both tetrads, one of the a segregants carries ste6::lacZ and the other carries STE6. The presence or absence of the mutant allele was determined by outcrosses for all four α segregants (data not shown). B-galactosidase activity in the MATa ste6::lacZ segregants is approximately 4.3 units, whereas B-galactosidase activity in α and a/α strains carrying the lacZ allele is undetectable above the background of the negative controls (less than 0.03 units). As expected, B-galactosidase activity is detectable in matα2 strains carrying the ste6::lacZ allele (data not shown), confirming the result

from RNA analysis that the α2 gene product is a negative regulator of STE6 expression.

From the B-galactosidase assays we estimate that expression of the fusion gene is at least 150-fold lower in α cells than in a cells. Interestingly, the ste6::lacZ gene is also fully regulated when carried on a high copy number plasmid: YEp13 carrying ste6::lacZ exhibits 150 units of B-galactosidase activity in an a cell and less than 0.03 units of activity in an α cell (K. Wilson, unpublished observations).

The orientation of the lacZ insertion indicates that the STE6 gene is oriented from right to left on the cloned DNA fragment as shown in Figure 2. We have shown that this orientation is correct by subcloning fragments into the single stranded DNA phage M13; we are able to protect the predicted strand from S1 nuclease digestion when the DNA is hybridized with RNA from a cells (K. Wilson, unpublished observations). No fragment is protected using RNA from α cells. This analysis places the 5' end of the STE6 transcript to the right of the KpnI site (K. Wilson, unpublished observations). The lacZ insertion is thus located approximately 1.2 kbp from the 5' terminus of the transcript.

DISCUSSION

The STE6 gene is required for mating by a cells but not by α cells. We have cloned STE6 and used it to study the function and regulation of STE6. A strain carrying the ste6::lacZ insertion mutation shows a large reduction in a-factor activity, which appears to be the sole reason for defective mating. STE6 is thus necessary for a-factor biosynthesis. Expression of the STE6 gene has been assayed in two ways: blot hybridization analysis of STE6 RNA and B-galactosidase activity produced by the STE6-lacZ hybrid gene. We find that expression of STE6 is limited to a cells and is negatively regulated by the α2 product of the α mating type locus.

The cloned segment contains the STE6 gene. STE6 was cloned by complementation of the mating defect of a MATa ste6 mutant. Evidence that the plasmid with complementing ability (YEp13-STE6) contains the STE6 gene is three-fold. First, the insert directs plasmid integration at the STE6 locus. Secondly, a DNA segment inserted into the cloned STE6 DNA by in vitro methods destroys the plasmid's complementing activity. Finally, replacement of homologous chromosomal sequences with this insertionally-altered DNA (ste6::lacZ) utilizing the one-step gene replacement method results in a recessive mutation tightly linked to the STE6 locus and unable to complement a ste6 defect. The ste6::lacZ insertion mutation alters a single α2-regulated RNA species, which is thereby deduced to be the STE6 transcript.

Function of the STE6 gene. The original ste6 mutant was isolated in a screen for mutants that are defective in mating (J. Rine, Ph. D.

thesis, University of Oregon, Eugene, 1979). Because this mutant maintains ability to respond to α-factor, it would not have been found in extensive mutant hunts in which non-mating mutants were selected for their resistance to α-factor (15, 26). The recessive ste6::lacZ insertion mutation produced in vitro causes a more severe mating defect than the original mutant allele, but it too affects mating only by a cells. The only known phenotype of these mutants, aside from their defect in mating, is that they are defective in a-factor activity. The mutant responds normally to α-factor: it arrests cell division and gives the characteristic altered cell morphology. Furthermore, the mutant agglutinates with α cells and exhibits Barrier activity (inactivation of α-factor). Because the only known defect of the ste6 mutant is in a-factor activity, it is likely that the STE6 gene is involved in biosynthesis of a-factor. The bioassay used to detect a-factor activity does not allow us to distinguish whether, for instance, the cells secrete low levels of normal a-factor or normal levels of altered a-factor (8). STE6 is unlikely to be a structural gene for a-factor because the STE6 restriction map does not correspond to the maps of two cloned a-factor genes (A. Brake, personal communication). The STE6 gene product might be necessary for expression of structural gene(s) for a-factor, for processing a putative precursor to a-factor, or for the secretion of a-factor.

It is striking that the ste6::lacZ mutant, despite a severe defect in mating due to the insertion mutation, still produces measurable a-factor (see Figure 3). Perhaps the insertion mutation allows formation of an amino-terminal segment of the STE6 protein that has partial

activity. It is also possible that the ste6::lacZ mutation is a complete inactivation of the STE6 gene but that another yeast gene product can partially substitute for STE6. More interesting possibilities are that unprocessed (or partially processed) a-factor precursor has some biological activity or that the STE6 product is not an absolute requirement for expression of a-factor genes.

Control of expression of STE6. Expression of the STE6 gene is limited to a cells: STE6 RNA is found in a cells but is not detectable in α or in a/α cells. A variety of genetic and physiological observations indicated that the α2 gene product of MATα is a negative regulator of genes involved in specialized a cell functions (34, 38). In particular, matα2 mutants are constitutive for several different a cell functions. We have shown that a matα2 mutation allows α cells to produce full levels of STE6 RNA. This result supports the previous inference that the α2 product is a negative regulator of certain a-specific genes and shows further that the α2 product acts to inhibit RNA synthesis from the STE6 gene. Because we are assaying stable RNA species, we are unable to distinguish whether α2 acts to inhibit transcription or affects RNA stability. Evidence that the α2 product is located in the nucleus (13) is consistent with the idea that the STE6 gene itself is the target of α2.

Studies with a ste6::lacZ fusion support the findings obtained with the hybridization assays. Our data show that the ste6::lacZ fusion, like the 4 kb RNA species, is regulated by α2 and hence that assay of B-galactosidase is a legitimate measure of expression of the

STE6 locus. B-galactosidase activity in a cells is approximately 4.3 units but is not detectable in α or a/α cells. We estimate from B-galactosidase activities that STE6 expression is at least 150-fold lower in cells containing the α2 product than in a cells. The ste6::lacZ fusion is also fully regulated when carried on a high copy number plasmid. These results indicate that negative regulation by α2 is potent. We note that assays of B-galactosidase activity might underestimate low level expression of the STE6 gene in α cells. Because B-galactosidase must tetramerize to be active (43), production of a low level of monomer as might occur in α cells would not be detected.

At least three genes are regulated by α2. In addition to STE6, expression of the BAR1 (A. Hartig and V. L. MacKay, personal communication) and STE2 (V. L. MacKay and T. R. Manney, personal communication) genes is limited to a cells and is inhibited by the α2 product. We anticipate that additional genes regulated by α2 will be identified.

The regulatory activities α1, α2, and a1-α2 have now all been demonstrated to regulate RNA synthesis (transcription or RNA stability) of their target genes: α1 is required for expression of STE3 (37) and MFα1 (R. Jensen, K. Wilson, and S. Fields, unpublished observations); and a1-α2 inhibits RNA synthesis from MATα1 (20, 29), HO (18), STE5 (V. L. MacKay, J. Thorner, and K. Nasmyth, personal communication), and the repeated element Ty1 (11). It is thus apparent that the mating type locus governs cell type in yeast by controlling synthesis of RNA for genes dispersed throughout the genome via its agents, α1, α2, and a1-α2.

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TABLE 1. STRAINS AND CROSSES

STRAIN	GENOTYPE	SOURCE
70	MATα thr3-10	F. Sherman
227	MATa lys1-1	J. Hicks
1369	MATα isogenic with HR125-5d	R. Jensen
G67A3	matα2-1/MATa STE6/STE6 (see reference 18)	G. Sprague
HR125-5d	MATa leu2-3 leu2-112 ura3-52 trp1-am his3 his4	R. Jensen
K39-3b	MATa ste6-21 (RSA21 allele of J. Rine) leu2-3 leu2-112 his4 ade6 lys2 can1	this work
K49-4b	MATα ste6-21 his4-am trp1-am ade2 ade6 lys2-oc tyr1-oc thr SUP4-3-ts	this work
K69-1d	MATa ste6-21 ura3-52 his4 lys2	this work
K72-33a	MATα ste6-21 ura3-52 leu2-3 leu2-112 aro2 lys5 ade5 his4 thr trp5	this work
K77	MATa ste6::lacZ; isogenic with HR125-5d	this work
K88-1, -2	MATa STE6::YIp5-STE6 (two independent Ura$^+$ integrants isogenic with HR125-5d)	this work
RC757	MATα sst2-1 met1 his6 can1 cyh2	R. Chan
VC2	matα1-2 ade6, other markers same as XP8-4a	V. MacKay
VC73	matα2-1 ade6, other markers same as XP8-4a	V. MacKay
XMB4-12b	MATa sst1-1 ilv3 arg9 ura1 Killer$^+$	L. Blair
XP8-4a	MATa leu1 trp5 his6 met1	P. Kushner
XP8-18b	MATα isogenic with XP8-4a	P. Kushner
XP11	MATa/MATα isogenic with XP8-4a	P. Kushner
XT1177-S245C	MATα ade6 his6 leu1 met1 trp5-1 gal2 can1 rme	T. Manney

TABLE 1. (Continued).

DIPLOIDS	PARENTS
K59	K77 X K49-4h
K61	K77 X 1369
K73-1, K73-2	K88-1 X K72-33a, K88-2 x K72-33a

TABLE 2

B-galactosidase activity in segregants from diploid K61

STRAIN	MAT	STE6	B-GAL ACTIVITY UNITS*
K61-2a	α	STE6	<0.03
2b	a	STE6	<0.03
2c	α	ste6::lacZ	<0.03
2d	a	ste6::lacZ	4.0
K61-3a	α	STE6	<0.03
3b	a	STE6	<0.03
3c	a	ste6::lacZ	4.7
3d	α	ste6::lacZ	<0.03
K61	a/α	STE6/ste6::lacZ	<0.03

* B-galactosidase assays performed as described in Materials and Methods.

FIG. 1. The α1-α2 hypothesis for control of cell type by the mating type locus. The structure and expression of the mating type locus (MAT) alleles in a, α, and a/α cells are shown (36) on the left. Unlinked genes whose expression is controlled by MAT are shown on the right. Wavy lines indicate gene expression. A line with an arrowhead represents stimulation of gene expression; lines ending in a bar represent inhibition of gene expression. αsg, α-specific genes; asg, a-specific genes; hsg, haploid-specific genes. Circled symbols indicate the regulatory gene products that carry out stimulation or inhibition as described in the text.

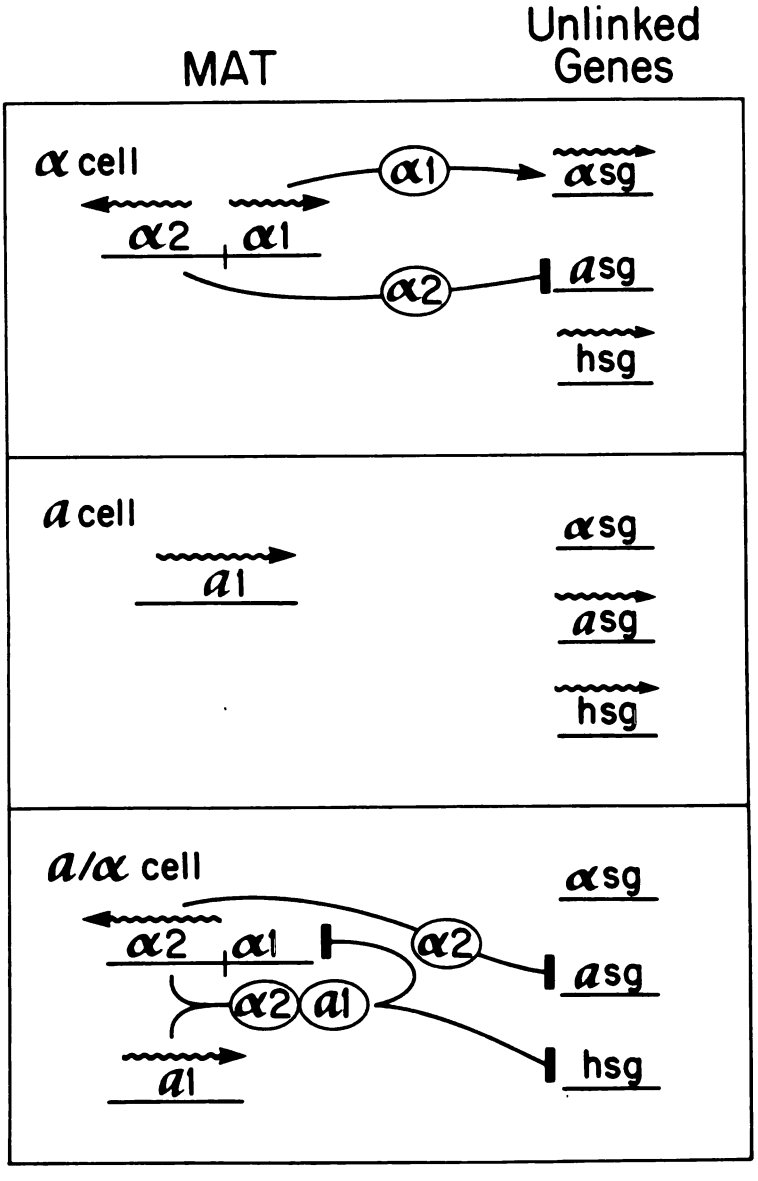
MAT
Unlinked Genes
α cell
α1
α2
α1
αsg
a sg
hsg
a cell
a1
αsg
a sg
hsg
a/α cell
αsg
α2
α1
α2
a sg
α2
a1
a1
hsg

FIGURE 1

FIG. 2. Restriction map of yeast DNA containing STE6, showing position of lacZ insertion. Shown is the restriction map of the original 5.5 kbp yeast DNA insert of YEpSTE6 drawn to physical scale. P, PstI; B, BamHI; E, EcoRI; H, HindIII; K, KpnI; S, SalI. Enzymes that do not cut within the yeast DNA include AvaI, BglII, ClaI, HindIII, HpaI, NaeI, PvuI, PvuII, SacI, SalI, SmaI, SphI and XhoI.

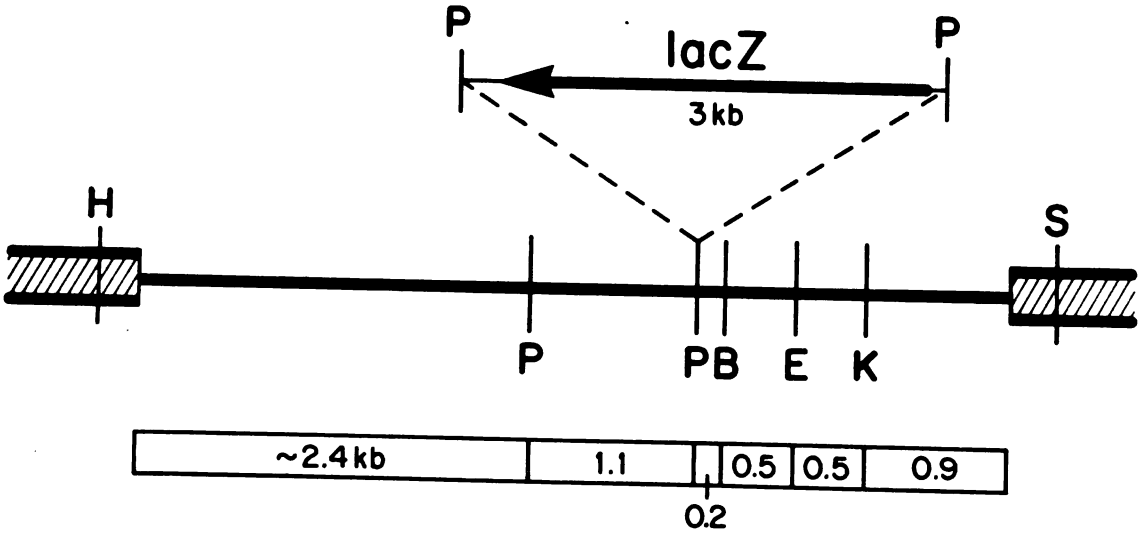

FIGURE 2

FIG. 3. Production of a-factor by a ste6::lacZ mutant. Strain K77 (carrying the ste6::lacZ mutation) and isogenic a and α strains (strains HR125-5d and 1369, respectively) are shown. Secretion of a-factor into the medium around a colony prevents growth of the supersensitive lawn (strain RC757), thus producing a "halo" or clear zone around the source of a-factor (see Materials and Methods).

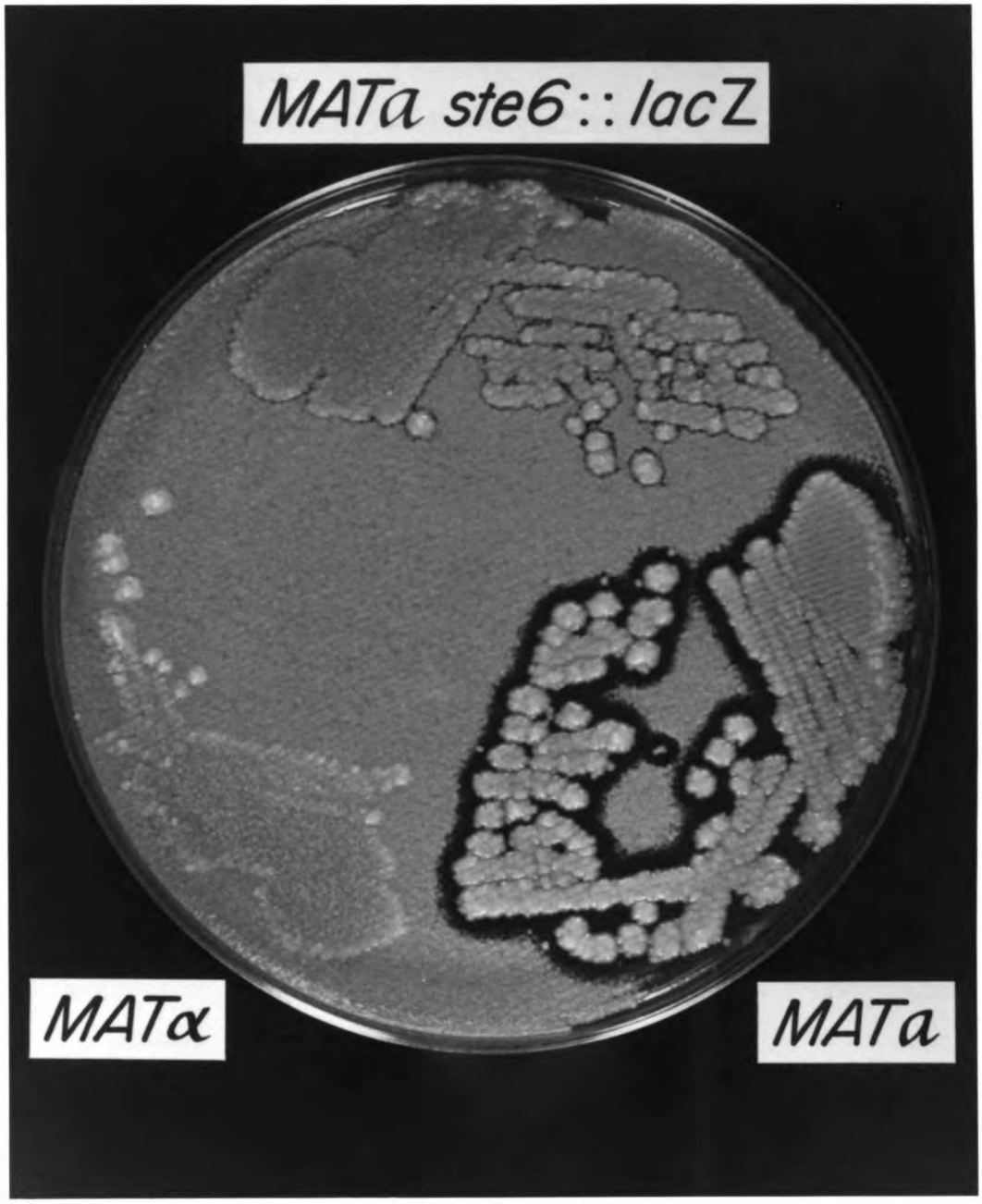

FIGURE 3

FIG. 4. Regulation of STE6 RNA production by the mating type locus. Poly(A)$^+$ RNA was isolated from a (XP8-4a), α (XP8-18b), a/α (XP11), matα2 (VC73) and matα1 (VC2) strains. 1.5 ug of RNA from each strain was fractionated by size by agarose gel electrophoresis, transferred to nitrocellulose paper, and hybridized with radioactively-labelled YIp5-STE6 plasmid DNA (see Materials and Methods). Positions of the STE6 and URA3 transcripts are indicated. The 3 kb transcript denoted "band X" is not related to STE6 (see text).

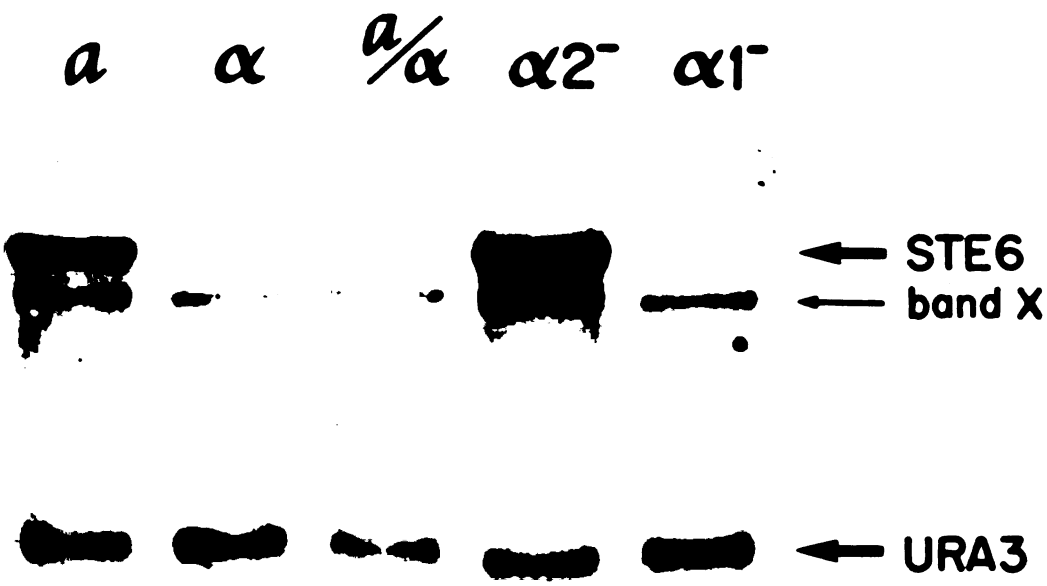

FIGURE 4

FIG. 5. Analysis of poly(A)$^+$ RNA from strain K77 (MATa, integrated ste6::lacZ gene) and a matα2/MATa diploid strain. The top and bottom panels are different exposures of the same gel. RNA was isolated and treated as described in the legend to Figure 4. Positions of the band X, STE6, URA3, and the truncated ura3-52 transcripts are indicated. Dots indicate the positions of new bands present in the ste6::lacZ strain. RNA from the following strains is shown:

Lane A, MATa STE6 strain XP8-4a

Lane B, MATa STE6 strain HR125-5d

Lane C, MATa ste6::lacZ strain K77
(K77 is isogenic with HR125-5d)

Lane D, matα2-1/MATa STE6/STE6 diploid
strain G67A3 (RNA from this strain was a gift
from R. Jensen).

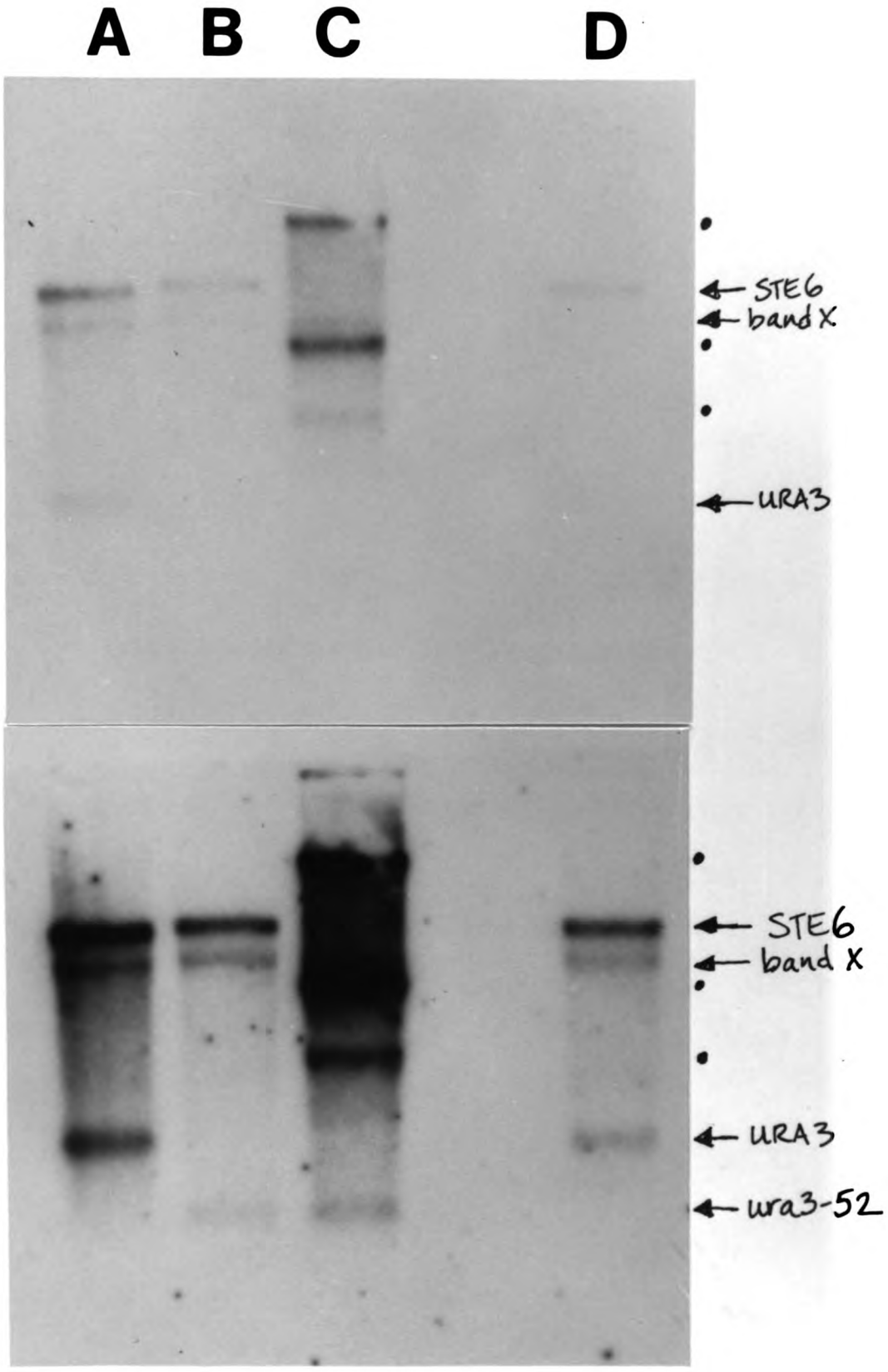

FIGURE 5

CHAPTER 3

A SITE UPSTREAM OF *STE6* IS REQUIRED FOR ITS REGULATION BY THE MATING TYPE LOCUS IN *SACCHAROMYCES CEREVISIAE*

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INTRODUCTION

Many eukaryotic genes recognized by RNA polymerase II are regulated at the level of transcription, by mechanisms that appear different from those used in procaryotes: the sites that regulate transcription initiation in eukaryotic genes are located away from the sites where transcription initiates (41, 55; reviewed in 56, 73). For a few well-studied genes in the yeast Saccharomyces cerevisiae, such upstream regulatory sites, or UASs, have been shown to mediate positive regulation by genetically-identified, specific regulatory proteins (12, 25, 35, 52, 60, 71; reviewed in 18). Negative regulation from upstream DNA has also been reported in yeast (1, 4, 57, 62, 70). However, direct interactions between negative regulatory proteins and the upstream sites through which they are proposed to act have not been demonstrated. We present evidence for an upstream repressor site required for the regulation of a cell-type-specific gene, STE6, by the yeast mating type locus, MAT.

Three regulatory activities are encoded by MATa and MATα (40, 66), the two alleles of MAT, as illustrated in Figure 3-1. MATa codes for one protein called a1, and the MATα allele codes for two proteins called α1 and α2 (51, 66). α1 is a positive regulator required for transcription of genes expressed only in α cells ("α-specific" genes), such as the STE3 gene (64). α2, in contrast, is a negative regulator that participates in inhibiting expression of two gene families. α2 alone inhibits transcription of genes required for a mating ability ("a-specific" genes such as STE6), while in the MATa/MATα diploid cell

the combined regulatory activity of α2 and a1, a1-α2, inhibits transcription of "haploid-specific" genes expressed in both a and α cells. Interestingly, a1-α2 inhibition of the MATα1 and HO genes is known to require sequences upstream of their respective transcription starts (57, 62). The mechanism of α2 transcriptional repression is not known either for α2 alone or for the a1-α2 activity.

We have examined simple negative regulation by α2 by studying the expression of the STE6 gene. STE6 gene function is necessary in a cells for the biosynthesis of a-factor pheromone, and a ste6$^-$ mutants are unable to mate (22, 72); hence the sterile designation. We showed previously that STE6 is regulated by the mating type locus: STE6 RNA is detectable in a cells, but not in α or a/α cells (72). Furthermore, the α2 product of the MATα locus is responsible for preventing STE6 transcription in α cells, since STE6 RNA is present in α cells defective in α2 activity. The BAR1 (V.L. MacKay and T. Manney, pers. comm.), STE2 (A. Hartig and V.L. MacKay, pers. comm.), and MFA1 (A. Brake, pers. comm.) genes are also repressed by α2. The genetic model proposed that STE6 and the other α2-regulated genes are expressed in the absence of the repressor α2.

To determine which DNA sequences around STE6 were required in cis for its regulation by α2, we constructed and analyzed a series of deletions that removed DNA upstream of a plasmid-borne ste6::lacZ fusion gene. We also determined the DNA sequence of the 5' terminal flanking region and presumptive N-terminal coding region of STE6, and located the STE6 transcript initiation sites.

We have defined a region approximately 150 basepairs upstream of the STE6 transcript initation sites that is required for regulation by α2 in vivo. We have shown that within this region that there exists a 31-basepair site that is conserved upstream of all sequenced genes known to be regulated by α2. We also present evidence for positive regulatory regions (a potential UAS) located upstream of this 31-basepair site.

MATERIALS AND METHODS

Strains, plasmids, and media

Yeast strains used were HR125-5d (MATa leu2-3 leu2-112 ura3-52 trp1-am his3 his4; from R. Jensen), 1369 (MATα; isogenic with HR125-5d; from R. Jensen), K77 (MATa ste6::lacZ; isogenic with HR125-5d; 72), XP8-4a (MATa leu1 trp5 his6 met1; from P. Kushner), XP8-18b (MATα; isogenic with XP8-4a; from P. Kushner), FC2-12b (MATα ura3-52 leu2-3 leu2-112 his4-401 trp1-am HOL1; obtained from R. Parker), and FC8-24d (MATa ura3-52 leu2 his4-401 trp1-am HOL1 actin intervening sequence deletion; obtained from R. Parker).

Escherichia coli strains used were MH6 (leuB600 pyrF::Tn5 lacX74 rpsL hsdR galU galK; obtained from M. Hall) and JM101 (Δlac,pro) supE thi traD36 proAB lacI^q ZΔM13).

Plasmids used were pBR322-ste6::lacZ (pBR322 carrying the ste6::lacZ fusion [72]) and two derivatives of YCp50 called YCp50-8 and YCp50-8-dBH, both constructed by K.L. Wilson. YCp50 is pBR322 containing URA3 CEN4 ARS1 (65; C. Mann, pers. comm.). YCp50-8 lacks the SmaI site of YCp50: YCp50 was cut with XmaI, which makes a staggered cut at the same site recognized by SmaI, then filled in and religated. YCp50-8-dBH lacks the pBR322 sequences between the HindIII and BamHI sites, but both sites are retained: YCp50-8 was cut with HindIII and BamHl, filled in with T4 DNA polymerase, and ligated with a HindIII linker d[CAAGCTTG]. The final G of the HindIII linker restored the BamHl site. The deletion version of YCp50-8 was constructed to avoid duplication of part of the Tet^r gene upon insertion of STE6 deletions (see Figure 3-2).

Phage M13mp8 and M13mp9 (46, 59), purchased from New England Biolabs, were used for sequencing.

Yeast medium SD-ura (synthetic complete medium plus glucose, lacking uracil) was prepared as described previously (24).

Genetic manipulations

Yeast transformations were done by the glusulase method (3, 27) using either gradient purified or miniprep DNA, or by the lithium method (29). E. coli transformations and DNA manipulations were performed by standard methods (42).

B-galactosidase assays

B-galactosidase assays were performed on late log phase cultures as described by Miller (47) with 50 ul of chloroform and 25 ul 0.1% sodium dodecyl sulfate used to permeabilize the cells. For each deletion plasmid-containing yeast strain shown in Table 3-2, a total of at least 5 assays were done using independent transformants. B-galactosidase activity was assayed over a period of up to four hours.

Deletion constructions

The strategy used to generate BAL31 deletions in the 5' flanking DNA of the STE6 gene is shown in Figure 3-2. BAL31 enzyme was purchased from Bethesda Research Laboratories. Plasmids were isolated from bacterial transformants that displayed the desired phenotype (Ura^+, blue color on medium containing the chromogenic substrate XG [5-bromo-4-chloro-3-indolyl-™-D-galactoside; Boehringer Mannheim Biochemicals]) and screened by restriction analysis for the extent of

BAL31 deletion and the orientation of the yeast insert within vector YCp50-8-dBH. Approximately 20 plasmids were transformed into isogenic a, α, and a/α yeast strains (HR125-5d, 1369, and their diploid). The effect of each deletion on B-galactosidase activity from the ste6::lacZ fusion was assessed by placing the yeast transformants on SD-ura plates containing XG. Based on these results a representative group of deletion plasmids was then subjected to individual HindIII digestion and religation to obtain plasmids carrying the deletion in each orientation. These constructs (listed in Table 3-1) were transformed into yeast and assayed for B-galactosidase activity (Table 3-2). In a given orientation, all deletion endpoints abut the same vector sequences, except deletion d2. DNA sequencing of the d2 endpoint revealed that the HindIII linker junction, normally vector-CAAGCTTG-STE6, had lost the final G nucleotide; the putative deletion endpoint is at position -103 in Figure 3-3, but since 5 bases of the linker are identical to those they replaced, the effective deletion endpoint is as shown at -109.

Sequencing strategy

The paucity of restriction sites in the relevant region of STE6 caused us to exploit the deletions constructed above for sequencing purposes. Figure 3-2 shows the strategy used. We subcloned restriction fragments (one end often being the HindIII site of a given deletion construction) into replicative (double-stranded) forms of phage M13mp8 and M13mp9. Sequencing reactions were carried out according to a protocol obtained from D. Peattie (personal communication; 2, 7) using α-^{35}S-dATP (Amersham, >600 Ci/mmol), except

that gels were dried directly after electrophoresis without soaking in acetic acid/methanol. The 17-mer sequencing primer d(GTAAAACGACGGCCAGT) (P-L Biochemicals) was used for all primary sequence determination. However, the Biolabs 15-mer primer d(TCCCAGTCACGACGT) was used to sequence most deletion endpoints. Dried-down gels were exposed at room temperature to Kodak XAR-5 X-Ray film for one to eight days. The complete sequence of both strands was obtained for the 593-basepair region defined at one end by the d31 deletion endpoint and at the other end by the STE6 HpaII site. The sequence data are presented in Appendix 2. All dideoxynucleotide termination mixes used were the generous gift of R. Parker.

S1 nuclease and primer extension mapping of the 5' ends of the STE6 transcripts

Polyadenylated (poly[A]) RNA from strains HR125-5d, 1369, K77, XP8-4a, and XP8-18b was isolated as described previously (72). Poly(A) RNA from strains FC2-12b and FC8-24d was a gift from R. Parker.

For S1 nuclease mapping we used M13mp9-K61, which contains the message sense strand of the 1.3 kilobasepair HindIII-BamHI restriction fragment of deletion d53 cloned in M13mp9, shown in Figure 3-6. ^{32}P-labelled hybridization probe was prepared as follows. Using single-stranded M13mp9-K61 phage DNA as template, DNA polymerase was used to extend a 17-mer sequencing primer across the inserted STE6 sequences in the presence of α-^{32}P-dCTP. The resultant double-stranded DNA was then cleaved with HindIII to give a labelled single-stranded fragment of approximate length 1.3 kilobasepairs that was cut out and eluted after electrophoresis through a 6% acrylamide/7M urea sequencing gel. 175 $g

of total (not poly(A)-enriched) RNA isolated from yeast strains XP8-4a and XP8-18b was hybridized with the single-stranded DNA probe. Hybridization and S1 digest conditions (S1 purchased from Bethesda Research Laboratories) and gel electrophoresis analysis were as described (51).

Primer extension analysis was carried out according to Domdey et al (14). For each reaction 5 §g of polyadenylated RNA was hybridized with primer (4×10^5 Cerenkov counts per minute) labelled at its 5' end using polynucleotide kinase (P-L Biochemicals). KW02, the 20-mer oligodeoxynucleotide primer (sequence shown in Figure 3-4) was synthesized and gel-purified by J. Barnett and C. Craik using a Beckman System 1 DNA Synthesizer. Life Sciences reverse transcriptase was then used to make cDNA in the presence of all four deoxyribonucleotides up to the 5' ends of the mRNA. The lengths of the cDNA products were determined by electrophoresis through 6% sequencing gels along with kinased size markers (HpaII-cut pBR328) and a Maxam-Gilbert purine sequence ladder of the rat insulin gene, donated by E. Fodor.

RESULTS

DNA sequence of a 593-basepair segment containing the STE6 5' flanking region, transcription initiation sites, and putative N-terminal coding region

A 5.5-kilobasepair DNA fragment was previously shown to contain the STE6 gene and sequences required for its regulation by α2 (72). In addition, we inferred the orientation of the STE6 transcription unit within the cloned DNA from the orientation of an α2-regulated ste6::lacZ gene fusion. In order to focus on potential regulatory regions, STE6 transcription initiation sites within the cloned DNA were roughly determined by S1 endonuclease protection analysis (described below). The sequence of a 593-basepair fragment that contained DNA adjacent to and including the 5' end of the STE6 gene was then determined by the Sanger dideoxy chain-termination method. The complete sequence of each strand was obtained from defined restriction fragments subcloned into the single-stranded phage M13. The restriction fragments used for sequencing were derived from a series of deletion constructions (described below) that placed HindIII endonuclease sites at convenient locations adjacent to the cloned DNA (Figures 3-2 and 3-3).

The complete sequence of the 593-basepair region at the 5' end of the STE6 gene is shown in Figure 3-4. The sequence predicts a single open reading frame (ORF) of 41 amino acids beginning at nucleotide +1, and shown in Figure 3-5. The location of the ORF with respect to the STE6 RNA start sites, and other factors discussed below, suggest that it constitutes the N-terminal 41 amino acids of the STE6 protein.

Location of the STE6 mRNA initiation sites

The general location of the 5' end of mature STE6 mRNA was determined by the S1 nuclease method of Berk and Sharp (6). A single-stranded, radioactively-labelled, 1.3-kilobasepair probe complementary to the predicted STE6 mRNA was isolated as described in Methods. As shown in Figure 3-7, when the probe is hybridized with RNA isolated from cells in which STE6 is expressed (MATa strain XP8-4a), DNA is protected from S1 digestion. A lighter exposure of the same gel revealed that two DNA fragments ca. 1040 and 1055 basepairs long were protected. No fragment was protected when the probe was hybridized with RNA from cells that do not express STE6 (MATα strain XP8-18b). This analysis confirmed our original observation that production of STE6 RNA is under mating type regulation and further indicated that there were at least two 5' ends of the STE6 mRNA, located approximately 360 basepairs downstream of the (d53)HindIII site.

More accurate determination of the number and positions of STE6 mRNA 5' ends was accomplished using the primer extension method of Domdey et al (14), described in Methods. A synthetic oligodeoxynucleotide 20 bases in length (oligo KW02, sequence 5'-CACGTACCTGAAAATGTGAT-3', see Figure 3-3), predicted by the DNA sequence and S1 analysis to be complementary to STE6 mRNA, was radioactively labelled at its 5' end and hybridized with poly(A) RNA from three different a strains and two different α strains. Once hybridized with STE6 RNA, the synthetic 20-mer served as primer for a reverse transcriptase reaction. The lengths of the end-labelled cDNA extension products were determined by electrophoresis on sequencing gels.

The primer extension analysis shown in Figure 3-8 revealed six cDNA species, corresponding to six different mature STE6 mRNA 5' ends clustered within a 25 basepair region. The RNA start positions are indicated as arrows in the DNA sequence (Figure 3-4). An error of plus or minus two bases is assumed. Thus the six 5' ends are located approximately 24 to 47 basepairs upstream of the first base of the presumptive STE6 translation initiation codon. The six cDNA species and hence the six RNA species were approximately equal in abundance; however, the cDNA corresponding to the RNA at position -28 was ca. twice as abundant as the others, in both strain backgrounds tested. In addition, with strain FC8-24d the cDNA corresponding to the longest RNA (initiation site -47) was as abundant as that initiating at position -28. No discrete primer extension products were seen when RNA from α cells was used. However, we note that a diffuse signal corresponding to initiation at positions -57 to -61 was present in the a HR125-5d strains and the α strain XP8-18b. Because this signal was absent when RNA from the FC8 strain background (either a or α cells) was used, we did not include it as a STE6 initiation site.

The S1 analysis described above suggested that no RNA processing occurs within 1000 basepairs of the 5' end of the ca. 4-kilobasepair STE6 transcript (or if it does, very little RNA is removed). RNA processing in yeast requires the sequences GTAYGT....TACTAAC (38, 53). The STE6 DNA sequence shows a consensus 5' splice junction (GTACGT) within the ORF at position +47, but there is no corresponding downstream TACTAAC within the sequenced DNA. Therefore, if this 5' splice site were used, we would expect a single DNA fragment of less than 925 basepairs (the approximate size of the KpnI-BamHI fragment) to

be protected from S1 digestion. In fact two fragments were protected that were at least 1040 bases long.

Deletions identify sequences required for cell type regulation of STE6

To determine whether DNA upstream of STE6 is required for α2 to inhibit transcription of STE6, we analysed a set of 5' flanking DNA deletions for their effect on STE6 expression. STE6 expression was assayed by measuring B-galactosidase activity from the ste6::lacZ fusion shown previously to be correctly regulated by α2 when integrated at the STE6 locus in the yeast chromosome (72). Furthermore, Figure 3-8 shows that RNA isolated from the chromosomally-integrated ste6::lacZ strain is the same as its wildtype counterpart in the number and position of RNA 5' ends capable of being primed with oligonucleotide KW02. However, for the deletion analysis all constructions were carried on a stable, low copy, yeast centromere-containing URA3 plasmid called YCp50-8-dBH (Figure 3-2).

We constructed a series of deletions using BAL31 endonuclease that left successively less and less DNA upstream of STE6, and joined each deletion endpoint via a synthetic HindIII linker to plasmid vector sequences as illustrated in Figure 3-2. Each deletion construction in both possible orientations was transformed into isogenic a and α yeast (ura3 strains HR125-5d and 1369) and grown in medium lacking uracil to select for maintenance of the URA3-containing plasmid. The effect of each deletion on STE6 expression was determined by measuring B-galactosidase activity from the yeast transformants. The results of the assays are presented in Table 3-2. The values given in Table 3-2 are the averages of assays from 5 or more independent transformants,

performed on 3 separate occasions. The conclusions for each set of assays with respect to its positive and negative controls were always the same. The negative control was α strain 1369 carrying no plasmid or carrying the plasmid vector without an insert. Strain K77, carrying the integrated ste6::lacZ fusion, was used as the positive control.

Table 3-2 presents the results of B-galactosidase assays for the deletion plasmids in both orientations. Examining first the data from plasmids in orientation I, two major conclusions can be reached. The first conclusion is that B-galactosidase activity from the ste6::lacZ fusion is properly regulated by the mating type locus when the fusion gene is carried on a plasmid. Hence, B-galactosidase activity was detectable in a cells but not in α cells when the deletion plasmid retained at least 239 basepairs of natural STE6 DNA upstream of the putative translation start (deletions d31, d61, d53, d62, d77, and d67). The second conclusion is that two deletions allow expression of B-galactosidase activity in both a and α cells (and in a/α diploids cells; data not shown). As shown by deletions d19 and d2, the removal of DNA sequences between positions -239 and -109 resulted in ste6::lacZ expression that was no longer inhibited by MAT. We have thus shown that a 130-basepair region of DNA upstream of the STE6 RNA initiation sites is required for inhibition of STE6 in MATα cells. Since we showed previously that the α2 protein inhibits STE6 expression at the level of transcription, we deduce from the deletion results that α2 requires specific DNA sequences within this 130-basepair upstream region in order to inhibit transcription of STE6.

The orientation of insertion of the ste6::lacZ DNA within its plasmid vector had an effect on the level of B-galactosidase

expression. Plasmids containing most or all of the starting DNA were not affected by orientation; B-galactosidase activity is detectable in a cells carrying deletions d31 and d61 in both orientations. However, with subsequent deletions d53, d62, d77, and d67, B-galactosidase activity in a cells is significant only from plasmids in orientation II. One explanation for this observation is that DNA sequences between -444 and -387 (see Figure 3-4) are required for efficient expression of the STE6 gene, and that vector sequences adjacent to the yeast DNA in orientation I can substitute for the lost STE6 DNA.

Sequence comparison between STE6 and other α2-inhibited genes indicates conservation of a 31-basepair site

STE6 is one of a family of genes whose transcription is inhibited by the α2 protein. If transcription inhibition by α2 is mediated by a specific DNA sequence, then such a sequence should be conserved among the different members of the α2-regulated gene family. We compared the 130-basepair region required for α2 regulation of STE6 with 5' DNA sequences of three other α2-regulated a-specific genes: STE2 (A. Burkeholder and L. Hartwell, personal communication), MFA1 (8), and BAR1 (V.L. MacKay, personal communication). All four sequences contain a conserved 31-basepair region within which exists a core where 22 of 27 bases are exactly the same in all four genes (allowing for one gap in the BAR1 sequence; see Figure 3-8). The sequence of the conserved region in STE6 is CATGTAATTACCTAATAGGGAAATTTACACG (indicated by a heavy line in Figure 3-4), located approximately 150 basepairs upstream of the RNA start sites. Thus, sequences shown by deletion analysis to be essential for α2 regulation of STE6 in vivo contain a 31-basepair site

that is conserved among all sequenced α2-regulated genes.

The STE6, BAR1, and MFa1 promoters may share additional functional components. In addition to being repressed by α2 they all require the STE12 gene product for maximal transcription (17), and expression of two (BAR1 [43] and a-factor [67]) is stimulated upon exposure of a cells to α-factor. We searched for sequence homology in the 5' flanking DNA of the three genes using the VUREPEATS program (44), restricting the search to perfectly conserved sequences at least 7 basepairs long. Four sequences in addition to the α2 binding site were found, shown in Figure 3-10. Curiously, sequences related to the STE6 α2 binding site exist in second locations upstream of the demonstrated (STE6) and putative (BAR1 and MFa1) α2 binding sites: the sequence AAAGGAAATT, similar to the right half of the α2 binding site, is found ca. 180 basepairs further upstream in BAR1; likewise elements that resemble the left half of the α2 binding site are found further upstream in MFa1 and STE6. The functional significance of these regions is under investigation.

DISCUSSION

Transcription of the STE6 gene is inhibited by the α2 product of the MATα locus (72). To approach the mechanism of repression by α2, we have identified regulatory sequences upstream of STE6 by deletion analysis. We determined the DNA sequence of a 593-basepair fragment comprising the upstream regulatory sequences, the STE6 transcription initiation sites, and the putative N-terminal coding sequence of the STE6 protein (Figure 3-4). We obtained two plasmid-borne 5' flanking DNA deletions that allowed constitutive expression of STE6, assayed by measuring B-galactosidase activity in yeast strains carrying deletions upstream of a ste6::lacZ fusion. The region removed by the constitutive deletions d19 and d2 contains a 31-basepair sequence found upstream of the four genes known to be regulated by α2. Because we showed previously that α2 regulates STE6 at the level of transcription, we deduce from the deletion results and the DNA sequence homology that α2 requires DNA sequences upstream of STE6 in order to inhibit transcription of STE6 in vivo. Our conclusions are complemented by biochemical evidence (32, 33) that the α2 protein itself binds DNA (specifically, the 31-basepair conserved site) in the region required for MAT regulation in vivo.

The putative N-terminus of the STE6 protein

The function of the STE6 protein is suspected to concern the biosynthesis or secretion of a-factor, the peptide mating phermone of a cells (63, 72). The STE6 protein has not been purified and its amino acid sequence is unknown. The DNA sequence reported here includes a small portion of the cloned STE6 locus and not the entire coding

sequence for the ca. 4-kilobasepair-long STE6 mRNA (72). We suggest that the open reading frame shown in Figures 3-4 and 3-5 codes for the N-terminus of the STE6 protein for the following reasons. First, the ATG putative initiation codon is the first ATG downstream of the STE6 RNA 5' termini. Current data indicate that translation initiates at the first AUG at the 5' end of the mRNA in eucaryotes (37, 61). Second, we deduce from the S1 nuclease analysis that no RNA splicing occurs within the 5' 1000 basepairs of the STE6 mRNA. (However, the possibility of downstream RNA processing cannot be ruled out). Finally, the other reading frames within the STE6 transcript contain multiple termination codons.

An unusual feature of the putative STE6 N-terminus is that the DNA sequence predicts a marked preference for rare amino acid codons as compared with the codon usage of genes that are expressed at high levels (5, 10). For example, the rare Arg CGG and Pro CCG codons are predicted (see Figure 3-5). In general, highly expressed yeast genes utilize only 25 of the possible 61 codons; this preference reflects the relative abundances of the tRNAs within yeast cells (5). In contrast to STE6 and the highly expressed (primarily glycolytic pathway) genes surveyed, the HO gene utilizes all amino acid codons with approximately equal frequency (except Arg CGG and Ser UCG which are not used) (57). While the apparent rare codon bias of STE6 may reflect a requirement for very low levels of the STE6 protein, and correlates with low mRNA abundance (72), it cannot be interpreted until the entire coding sequence and function of STE6 are known. We note that the final fourteen amino acid residues are devoid of charged residues, while charged residues are scattered throughout the preceeding twenty-seven

residues. We cannot predict whether the hydrophobic portion indicates a transmembrane segment, an interior part of the protein, or a signal peptide.

The STE6 transcripts initiate at multiple sites

Northern analysis indicated that the STE6 transcript is at least 4 kilobasepairs long (72). From S1 protection and primer extension analysis we have determined the 5' (but not the 3') limits of the STE6 transcripts, which correspond to the upper strand of Figure 3-4. There were six RNA initiation sites clustered within a 26 basepair region located approximately 23 basepairs upstream of the presumptive translation initiation codon (Figures 3-4 and 3-8). The longest RNA initiates at the sequence CAAG approximately 47 basepairs prior to the ATG; the sequence YAAG is found near RNA start sites of several yeast genes (10, 13, 58). The more abundant STE6 transcripts initiate at -28 and -47. The methods used to examine the 5' termini of the STE6 transcripts cannot distinguish between primary transcripts and stable modified or degraded transcripts. However, other yeast genes examined by these methods and reported to initiate transcription at multiple sites include CYC1 (16), GAL10 (21), HIS1 (26), MAT (51, 62), and SUC2 (11). Interestingly, changes in the frequency of initiation at a subset of sites have been observed upon derepression (11) or down-regulation (51) of some loci; for example, derepression of the SUC2 locus correlates with the selective increase in abundance of the longer mRNA encoding the secreted form of invertase. We know that the six STE6 RNA species were absent in α cells, and therefore that all responded equally to regulation by α2.

Specific features of the 5' region

The canonical sequence TATAAAA (TATA box; M. Goldberg, Ph.D. thesis, Stanford University, Palo Alto, 1979) is a gene proximal element shown in higher eucaryotes to be essential for accuracy and efficiency of transcription initiation in genes recognized by RNA polymerase II (45; reviewed in 9). In bacteria the homologous "Pribnow box" (5' TATAAT 3'; 54) is contacted by bacterial RNA polymerase (34), and this is thought to fix the transcription initiation site. In yeast, deletions of TATA sequences do not alter regulation of a locus (regulation evidently being determined by sites further upstream), but greatly reduce levels of transcription (20, 69). TATA sequences appear to determine initiation sites in vitro in higher enkaryotes (see 9) and in vivo in yeast (16, 62). Yeast genes generally have one or more TATA boxes 40-70 basepairs upstream of the transcript initiation site(s) (58). There are three potential TATA boxes for STE6: a good match (TATAAA at position -100, ca. 60 basepairs upstream of the longest RNA) and two imperfect ones (TAAATA at ca. -115 and TATACAA at -165) (Figure 3-4 and 3-8). We have no evidence indicating which of the TATA sequences is biologically relevent, except to note that deletions d19 and d2 both retain the "good" TATAAA.

Regulation of STE6 by α2 requires upstream sequences

We obtained deletions that affected both the expression of STE6 and its regulation by mating type. The effect of the deletions on mating type regulation are best seen by examining data from the deletions in orientation I within the vector. Ste6::lacZ expression is properly regulated by cell type when the plasmid carries at least 239

basepairs of natural DNA upstream of the putative STE6 translation initiation site: B-galactosidase activity from deletion plasmid d67 was detectable only in a cells. However two deletions caused cell type-independent expression of the fusion gene: B-galactosidase activity was detectable in both a and α cells carrying deletions d19 and d2. Neither deletion removed DNA corresponding to STE6 RNA. Therefore, deletions d67 and d19 define a 130-basepair region upstream of STE6 that is required for cell type regulation of STE6 expression. Since we know that α2 regulates STE6 at the level of transcription, sequences within this 130-basepair region must be required for α2 to inhibit transcription.

We compared the sequence of the 130-basepair upstream region shown by deletion analysis to be required for α2 regulation of STE6 with the sequences of three other α2-regulated genes: STE2, BAR1, and MFa1. We found a conserved 31-basepair site that is located 181 to 233 basepairs upstream of each coding sequence. As shown in Figure 3-9, the sequence of the conserved site in STE6 is

5'-CATGTAATTACCTAATAGGGAAATTTACACG-3'.

Johnson et al (32, 33) have shown that the α2 protein is a repressor that binds to the 31-basepair site and protects it from nuclease digestion. Our deletions show that the operator site bound by the repressor in vitro is biologically significant, since loss of the site allows unregulated, constitutive expression in a and α cells (and a/α cells; data not shown). The α2-binding site functions in either orientation, as the site upstream of the STE2 gene is inverted compared to the sites upstream of STE6, BAR1, and MFA1 (Figure 3-9).

The consensus α2-binding sequence among the four α2-regulated genes may represent a recognition site for the solo α2 regulatory activity:

5' CATGTAATTACCNAA AAGGAAATTTACATG 3'

3' GTACATTAATGGNTT TTCCTTTAAATGTAC 5'.

The consensus site is symmetric about the six central basepairs, hinting that α2 may bind as a dimer. Interestingly, a sequence related to the α2 site has been identified by Siliciano as necessary for a1-α2 repression of MATα1 (Figure 3-10B; 62). It is possible that STE6 expression in a/α diploid cells is repressed by the a1-α2 activity, in addition to or instead of the α2 activity. Curiously, there are sequences strikingly related both to the α2 binding site and to the site required for a1-α2 regulation located further upstream of the α2 consensus site in STE6, MFA1, and BAR1 (see Figure 3-10A).

Expression of STE6 requires a site upstream of the α2-binding site

Observation of deletion effects on STE6 expression (assayed as B-galactosidase activity) depended upon the orientation of the insert ste6::lacZ DNA within its plasmid vector. Deletions d31 and d61, which removed the least DNA, were expressed in a cells regardless of their orientation within the vector. However, deletions that removed more DNA (deletions d53, d62, d77, d67, d19, and d2) were expressed significantly in yeast only in orientation I. Every deletion abuts the same vector sequences in a given orientation (except deletion d2; see Methods and Figure 3-2).

One explanation for the orientation dependence is that yeast sequences between -444 and -388 (Figure 3-3) are required for autonomous STE6 expression and that vector sequences adjacent in orientation I but not orientation II are capable of substituting for the missing DNA. A similar observation concerning orientation within the YCp50 plasmid was made with an HO-lacZ fusion (57). The pBR322 sequences responsible for promoting expression of nearby yeast genes are located in the vicinity of the BamHI site, on the SalI side (see Figure 3-2), and have been noted by Struhl (68).

We suggest that STE6 has an upstream activation site or UAS located upstream of the α2 binding site, based on the orientation-dependence of STE6 expression seen with our deletion constructions. There is a candidate for a positive regulator of α2-inhibited loci: the STE12 gene product is necessary for full levels of STE6 RNA in a cells, since STE6 RNA was approximately 3-fold reduced in abundance in a ste12 cells (17). The same mutant ste12 allele had more dramatic effects (up to 50-fold reduction) on the levels of RNA from other genes. STE12 is formally a positive regulator of two classes of mating-related genes: the a-specific α2-repressed genes and the α-specific α1-activated genes (17). A sequence comparison between the STE6 proposed UAS region, BAR1 and MFA1 (two other a-specific genes), yielded sequence homologies other than the α2 binding site that have not yet been tested for function (Figure 3-10). Sequences within the 130-basepair region that contains the α2 binding site also contribute positively to STE6 expression, since deletions d19 and d2 also abolish the vestiges of STE6 expression seen in a cells carrying deletions d53, d62, d77, and d67 in orientation II (Table 3-2).

We thus distinguish three regions within the STE6 promoter: first, an upstream region required for expression (potential UAS); second, the 31-basepair site required for α2 repression; and third, the TATA boxes and transcription initiation sites. Our evidence indicates that repression of STE6 occurs at the level of stable transcript initiation and not RNA stability, since the site required for repression is located well upstream of where transcription initiates. Furthermore we have demonstrated that an upstream region required for expression (the potential UAS) is separate from the site that mediates mating type repression (the α2 operator). This situation is in contrast to positive regulation, where UASs are coincident with the sites required for specific activation. It is not known where RNA polymerase II gains entry to transcribed loci in yeast or how this relates to the mechanism(s) of UAS action. However, the separation of positive and negative regulatory sites does not allow us to distinguish between mechanisms wherein represser inhibits the progression of transcriptional complexes along the DNA, and negative effects on the initial binding of polymerase to the promoter. Positioning of the α2 site between the UAS and the TATA/transcript start, as we propose is the case for STE6, is not necessarily required for α2 to function: significant repression by α2 still occurs when the 30-basepair STE6 α2 site is placed upstream of the CYC1 UAS on a high copy plasmid (33). The results described here and by Johnson et al (32, 33) combine to provide direct evidence for transcriptional repression via an upstream site, in determining the specialized a cell type in yeast.

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TABLE 3-1. Deletion plasmids

Deletion name	Orientation I	Orientation II
d31	pC6L-d31	pC6L-d31a
d61	pC6L-d61-1	pC6L-d61
d53	pC6L-d53-3	pC6L-d53-5
d62	pC6L-d62	pC6L-d62-3
d77	pC6L-d77-2	pC6L-d77-3
d67	pC6L-d67-2	pC6L-d67-3
d19	pC6L-d19b	pC6L-d19a
d2	pC6L-d2a	pC6L-d2f

TABLE 3-2. Effect of upstream deletions on regulation and expression of the ste6::lacZ fusion

Deletion name	Deletion endpoint	B-galactosidase Activity (Miller Units)			
		Orientation I		Orientation II	
		a	α	a	α
d31	-469	4.0	0.07	3.1	0.07
d61	-444	1.8	0.07	2.0	0.05
d53	-388	2.6	0.05	0.15	0.02
d62	-346	6.3	0.04	0.13	0.02
d77	-255	8.0	0.06	0.10	0.05
d67	-239	5.1	0.05	0.35	0.03
d19	-109	0.9	1.3	0.04	0.06
d2	-108	0.7	1.0	0.06	0.07

YCp50-8 in HR125-5d (vector only): 0.03
Yeast strain K77 (a ste6::lacZ): 7.7

Legend to Figure 3-1. The α1-α2 hypothesis for control of cell type by the mating type locus. The structure and expression of the mating type locus (MAT) alleles in a, α, and a/α cells are shown on the left. Unlinked genes whose expression is controlled by MAT are shown on the right. Wavy lines indicate gene expression. A line with an arrowhead represents stimulation of gene expression; lines ending in a bar represent inhibition of gene expression. αsg, α-specific genes; asg, a-specific genes; hsg, haploid-specific genes. Circled symbols indicate the regulatory gene products that carry out stimulation or inhibition as described in the text.

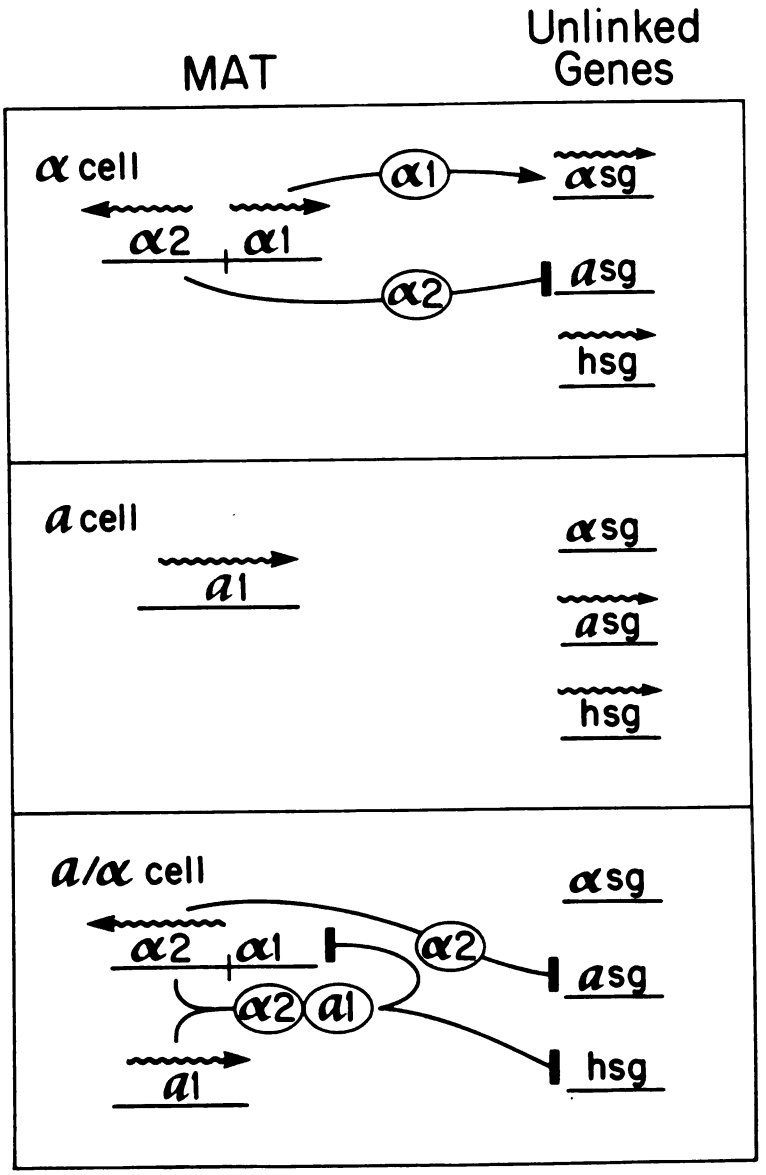

MAT
Unlinked Genes
α cell
α1
αsg
α2
α1
asg
α2
hsg
a cell
a1
αsg
asg
hsg
a/α cell
αsg
α2
α1
α2
asg
α2
a1
hsg
a1

FIGURE 3-1

Legend to Figure 3-2. Deletion strategy.

This figure shows the construction of deletions that removed DNA upstream of the ste6::lacZ fusion gene. On the left: the strategy for BAL31 digestion, end-repair, and ligation to HindIII linkers. On the right: construction of the derivative of plasmid YCp50-8 that has a portion of the tet^R gene removed. Note that the deleted tet^R segment is replaced either forward or backward upon insertion of the DNA fragment carrying the ste6::lacZ fusion, depending on the orientation of insertion of the ste6::lacZ HindIII fragment.

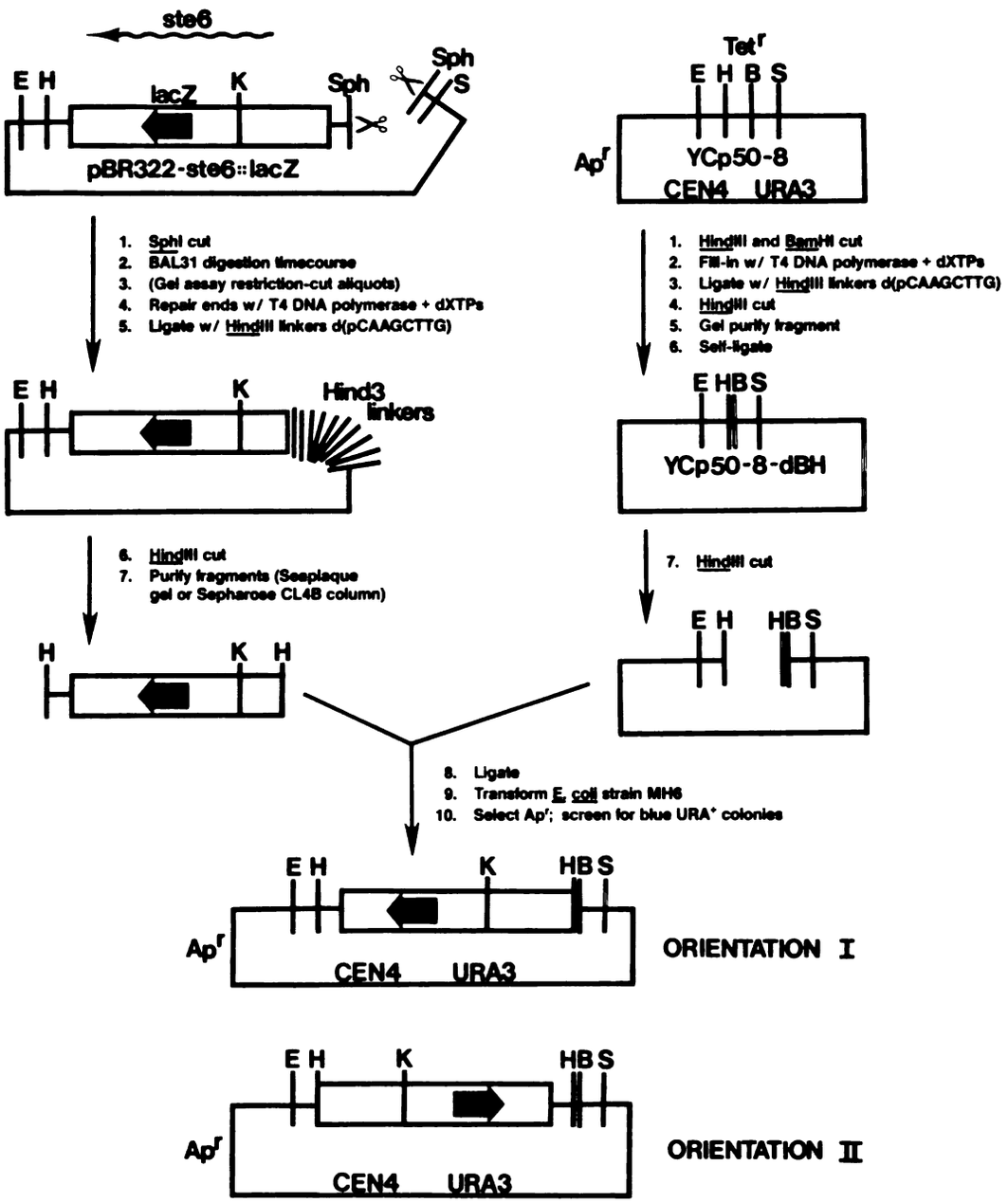


FIGURE 3-2
105

Legend to Figure 3-3. DNA sequencing strategy.

The top line depicts the 593-basepair sequenced DNA fragment. Restriction endonuclease sites are abbreviated as follows: A= AhaIII; R= RsaI; HpaII as stated. Where a deletion endpoint is indicated, for example d62, a HindIII site exists in that particular deletion construction, bounded on the left by plasmid vector sequences. Therefore plasmids carrying deletion d62 retain STE6 DNA to the right of the d62-HindIII site, but not to the left.

The arrows represent restriction fragments subcloned into M13 phage vectors mp8 and mp9 using the restriction sites indicated at the ends of each arrow ("d62", for example, refers to the HindIII site present in the d62 plasmid). If the arrow points to the right, then the fragment was cloned in mp8 and the sequenced strand corresponds to the upper strand of Figure 3-4. If the arrow points left, then the fragment was cloned in mp9 and the sequenced strand corresponds to the complementary lower strand of Figure 3-4. The complete sequence of both strands was determined.

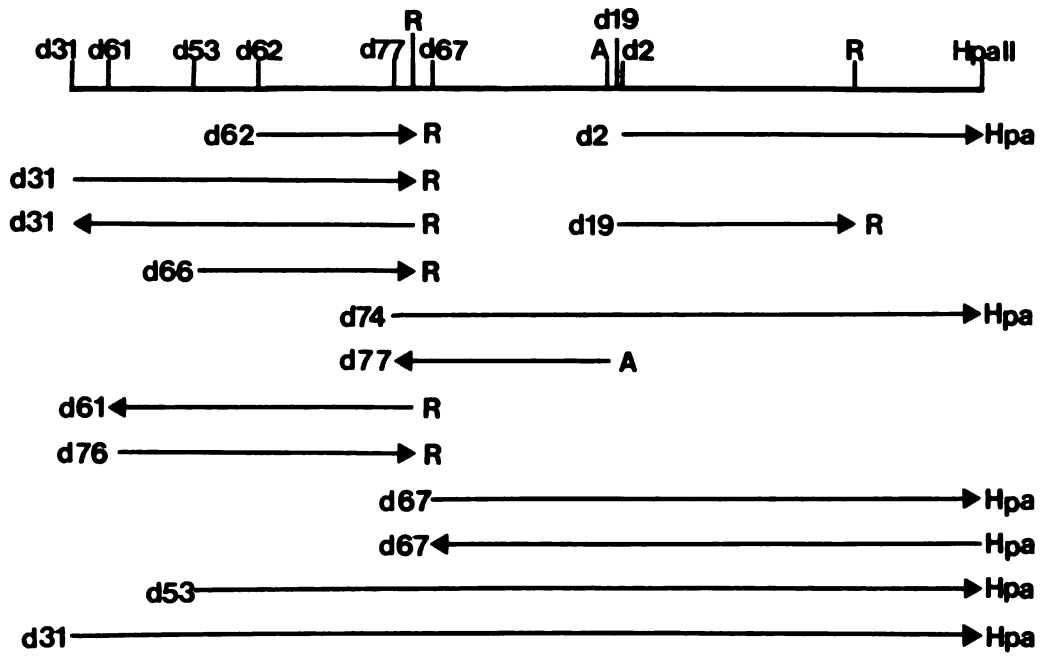

R
d19
d31
d61
d53
d62
d77
d67
A
d2
R
HpaII
d62
R
d2
Hpa
d31
R
d31
R
d19
R
d66
R
d74
Hpa
d77
A
d61
R
d76
R
d67
Hpa
d67
Hpa
d53
Hpa
d31
Hpa

FIGURE 3-3

Legend to Figure 3-4. The DNA sequence of a 593-basepair fragment comprising the 5' flanking and putative N-terminal coding sequence of STE6.

Shown is the double-stranded sequence of the STE6 promoter region. d31, d61, etc., refer to BAL31-generated deletion endpoints constructed as shown in Figure 3-2. A given deletion construction retains all STE6 sequences to the right of the indicated deletion endpoint, and is flanked on the left by a HindIII linker (5' CAAGCTTG 3') and vector sequences.

Nucleotides are numbered with respect to the A of the putative translation inititation codon (+1) which begins the open reading frame (ORF) indicated in the figure. The 31-basepair region that is homologous with other α2-regulated genes (α2 operator site) is indicated by a dark bar. RNA start sites determined by primer extension (Figure 3-7) are indicated as stubby arrows; the dark arrow indicates the RNA species of relatively higher abundance. Potential TATA boxes are marked with hollow diamonds. Restriction sites are indicated except for an AluI site at -380. The single-stranded oligonucleotide used for primer extension analysis is indicated as an arrow marked KW02.

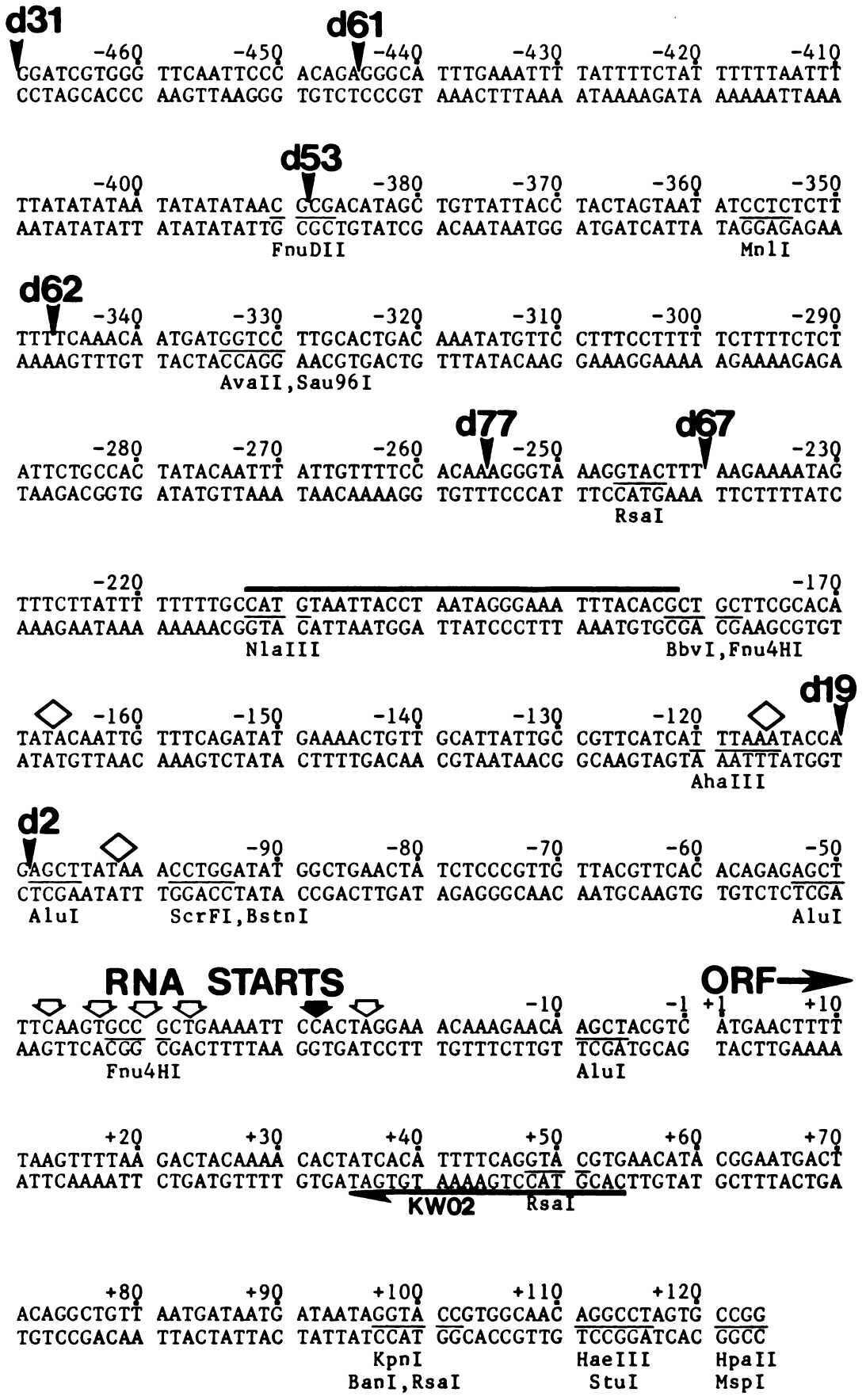
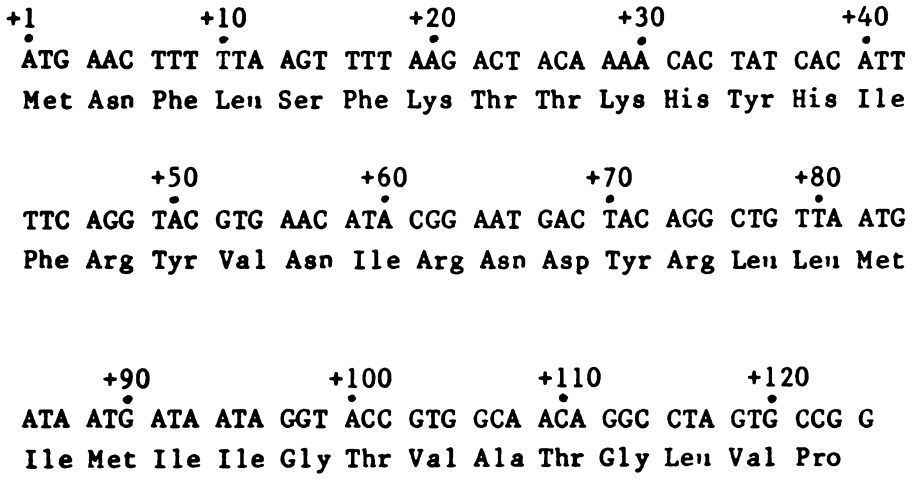
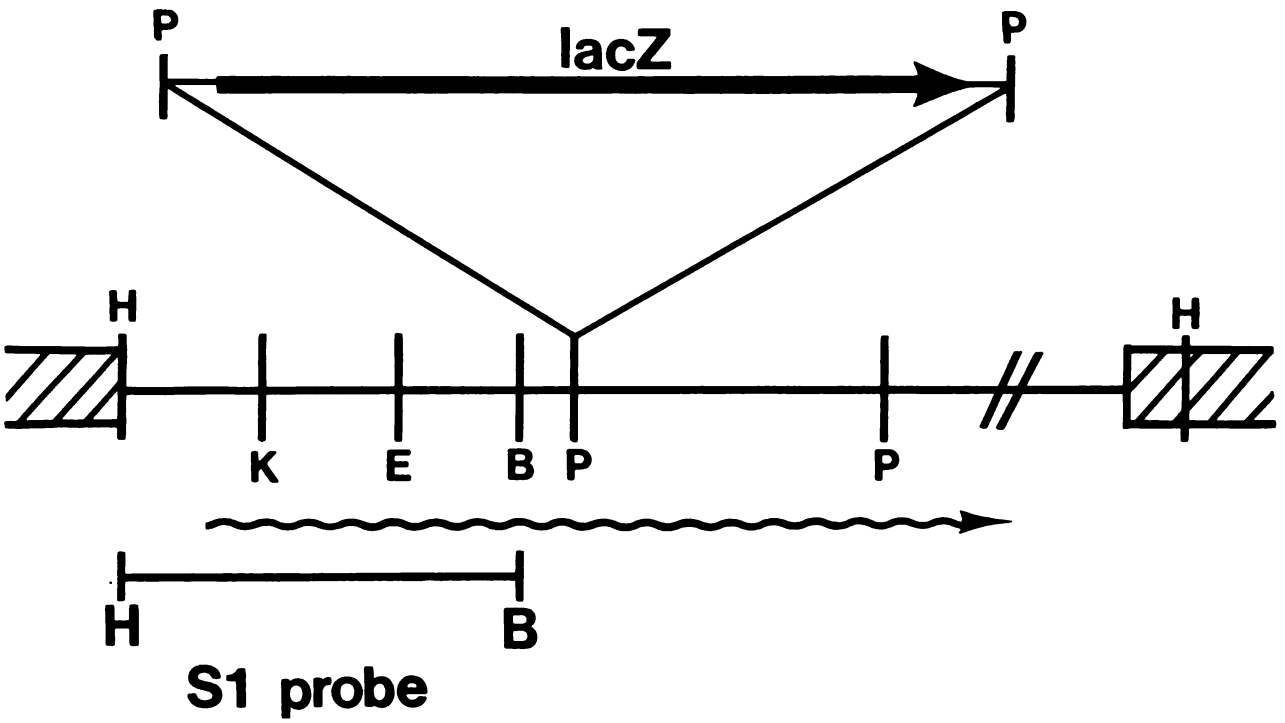

d31
d61
-460 -450 -440 -430 -420 -410
GGATCGTGGG TTCAATTCCC ACAGAGGGCA TTTGAAATTT TATTTTCTAT TTTTTAATTT
CCTAGCACCC AAGTTAAGGG TGTCTCCCGT AAACTTTAAA ATAAAAGATA AAAAATTAAA
d53
-400 -380 -370 -360 -350
TTATATATAA TATATATAAC GCGACATAGC TGTTATTACC TACTAGTAAT ATCCTCTCTT
AATATATATT ATATATATTG CGCTGTATCG ACAATAATGG ATGATCATTA TAGGAGAGAA
FnuDII
MnlI
d62
-340 -330 -320 -310 -300 -290
TTTTCAAACA ATGATGGTCC TTGCACTGAC AAATATGTTC CTTTCCTTTT TCTTTTCTCT
AAAAGTTTGT TACTACCAGG AACGTGACTG TTTATACAAG GAAAGGAAAA AGAAAAGAGA
AvaII,Sau96I
d77
d67
-280 -270 -260 -250 -230
ATTCTGCCAC TATACAATTT ATTGTTTTCC ACAAAGGGTA AAGGTACTTT AAGAAAATAG
TAAGACGGTG ATATGTTAAA TAACAAAAGG TGTTTCCCAT TTCCATGAAA TTCTTTTATC
RsaI
-220 -170
TTTCTTATTT TTTTTGCCAT GTAATTACCT AATAGGGAAA TTTACACGCT GCTTCGCACA
AAAGAATAAA AAAAACGGTA CATTAATGGA TTATCCCTTT AAATGTGCGA CGAAGCGTGT
NlaIII
BbvI,Fnu4HI
d19
-160 -150 -140 -130 -120
TATACAATTG TTTCAGATAT GAAAACTGTT GCATTATTGC CGTTCATCAT TTAAATACCA
ATATGTTAAC AAAGTCTATA CTTTTGACAA CGTAATAACG GCAAGTAGTA AATTTATGGT
AhaIII
d2
-90 -80 -70 -60 -50
GAGCTTATAA ACCTGGATAT GGCTGAACTA TCTCCCGTTG TTACGTTCAC ACAGAGAGCT
CTCGAATATT TGGACCTATA CCGACTTGAT AGAGGGCAAC AATGCAAGTG TGTCTCTCGA
AluI
ScrFI,BstnI
AluI
RNA STARTS
ORF
-10 -1 +1 +10
TTCAAGTGCC GCTGAAAATT CCACTAGGAA ACAAAGAACA AGCTACGTC ATGAACTTTT
AAGTTCACGG CGACTTTTAA GGTGATCCTT TGTTTCTTGT TCGATGCAG TACTTGAAAA
Fnu4HI
AluI
+20 +30 +40 +50 +60 +70
TAAGTTTTAA GACTACAAAA CACTATCACA TTTTCAGGTA CGTGAACATA CGGAATGACT
ATTCAAAATT CTGATGTTTT GTGATAGTGT AAAAGTCCAT GCACTTGTAT GCTTTACTGA
KW02
RsaI
+80 +90 +100 +110 +120
ACAGGCTGTT AATGATAATG ATAATAGGTA CCGTGGCAAC AGGCCTAGTG CCGG
TGTCCGACAA TTACTATTAC TATTATCCAT GGCACCGTTG TCCGGATCAC GGCC
KpnI
HaeIII
HpaII
BanI,RsaI
StuI
MspI

FIGURE 3-4

FIGURE 3-5. Amino acid sequence of putative STE6 N-terminus





Legend to Figure 3-6. S1 mapping strategy.

The 1.3 kilobasepair HindIII-BamHI fragment from deletion construction d53 cloned into M13 phage (M13mp9-K61) was radioactively labelled and hybridized with total RNA from a and α yeast strains as described in Methods. The 5' but not the 3' limits of the RNA were determined. Restriction sites are abbreviated as follows: B= BamHI; E= EcoRI; H= HindIII; K= KpnI; P= PstI. Vector sequences are indicated as hatched bars. The STE6 transcript is indicated as a wavy line; the arrowhead indicates the 3' end of the transcript.

Legend to Figures 3-7A and 3-7B. S1 mapping the 5' ends of STE6 mRNA.

The details of the S1 analysis are described in Methods. Size markers were kinased HaeIII fragments of OX174, indicated at left by bars. The 1.3 kilobase single-stranded radioactive DNA probe (see Figure 3-6) was hybridized with total RNA from isogenic strains HR125-5d (MATa) and 1369 (MATα), digested with S1 nuclease, and electrophoresed through acrylamide. Two exposures of the same gel are shown. The overexposed left panel shows that the α strain has no RNA corresponding to STE6. The underexposed right panel shows that at least two DNA species corresponding to at least two STE6 RNAs are protected from S1 digestion using RNA from a cells.

Figure 3-7B. To better determine the sizes of the protected fragments, S1-protected sample from the a strain described above was run on a new gel by itself (Lane "a"), and after mixing with the size markers (Lane "a+m"). Lane P is undigested full-size probe (arrowhead). The two protected fragments and their estimated sizes are indicated by arrows.

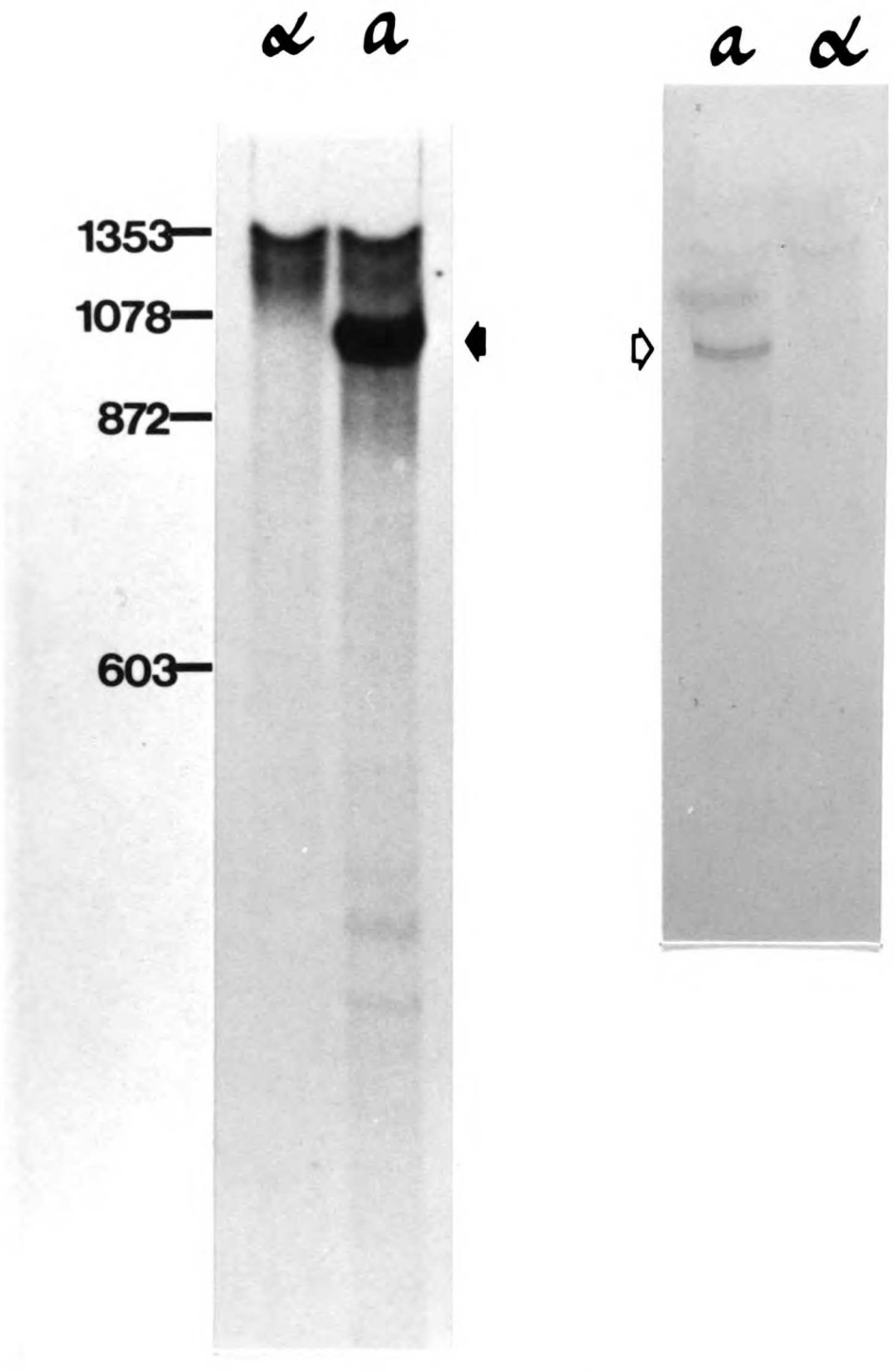


FIGURE 3-7A

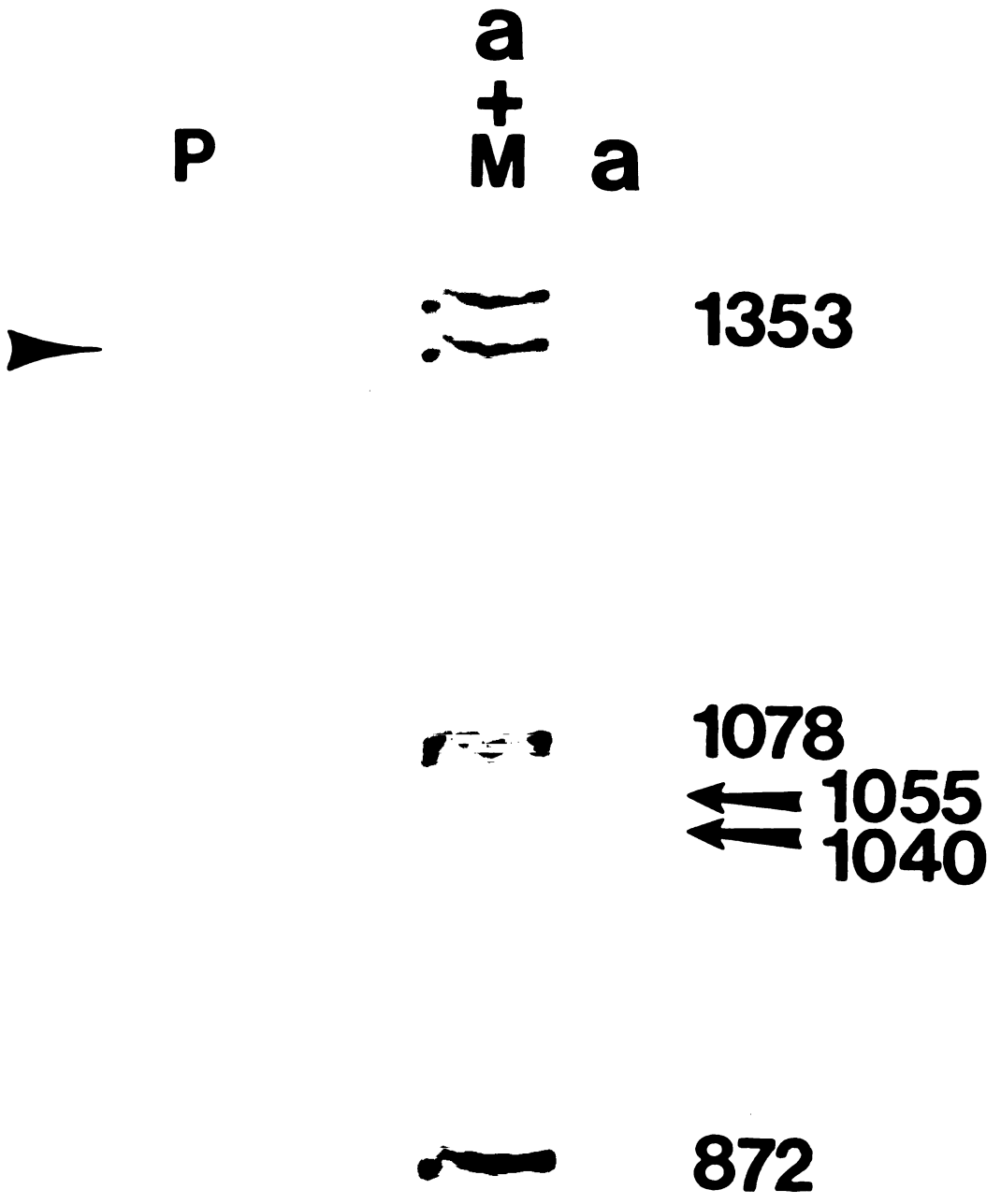


FIGURE 3-7B

Legend to Figure 3-8. Primer extension analysis of STE6 mRNA.

Primer extension was done as described in Methods. Lanes "m" are kinased HpaII fragments of pBR325 used as size markers; fragment lengths (nucleotides) are indicated. Lane F is a Maxam-Gilbert purine sequence ladder of the rat insulin gene. The other lanes contain reverse-transcribed extension products from oligonucleotide KW02 hybridized to poly(A)$^+$ RNA isolated from the following strains:

Lane A: MATα STE6 strain 1369

Lanes B: MATa ste6::lacZ strain K77

Lanes C: MATa STE6 strain HR125-5d

(1369, K77, and HR125-5d are isogenic)

Lane D: MATa strain FC8-24d

Lane E: MATα strain FC2-12b

The left and right autoradiographs represent identical samples run on different gels. On the right, stars indicate cDNA bands that correspond to the 5' ends indicated in Figure 3-4 as RNA start sites. A vertical bar indicates a diffuse signal that was not counted as a genuine start site.

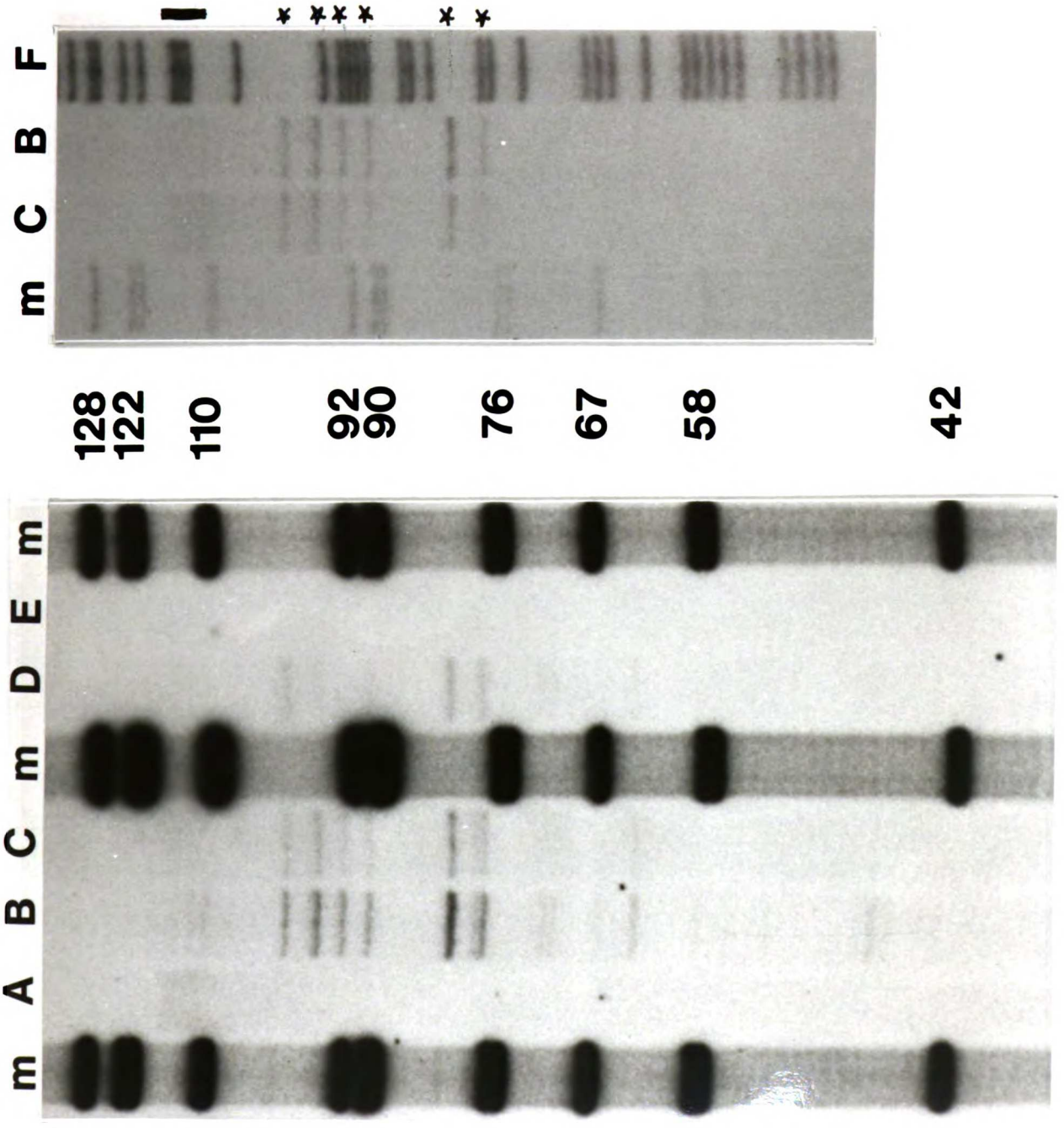


FIGURE 3-8

Legend to Figure 3-9. The 31-basepair sequence homology between STE6 and three other α2-regulated genes.

Panel A shows the sequence of a 31-basepair site found in the region required for MAT regulation of STE6 that is also found upstream of STE2, BAR1, and MFA1. In the consensus sequence, perfect homology among all four genes is indicated by underlining; bases not underlined are present in three of the four sequences. N means any base. As depicted in the Discussion, the consensus site is symmetric about the central six basepairs. The T missing from the BAR1 sequence (indicated as a gap) causes the symmetry to be better in the BAR1 site than in the others.

Panel B shows the location of the 31-basepair site upstream of each gene with respect to its translation initiation codon. Transcription initiation sites have not yet been determined for STE2, BAR1, or MFA1. Note that the 31-basepair site upstream of STE2 is oriented opposite to the others.

A

Gene	Sequence
STE6	CATGTAATTACCTAATAGGGAAATTTACACG
STE2	CATGTACTTACCCAATTAGGAAATTTACATG
BAR1	CATGTAATTACCGAAAAAGGAAATT ACATG
MFA1	TGTGTAATTACCCAAAAAGGAAATTTACATG
consensus	CATGTAATTACCNAANAAGGAAATTTACATG

B

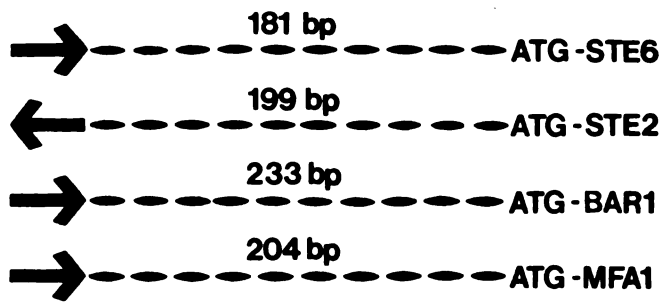


FIGURE 3-9

Legend to Figure 3-10. DNA sequence homologies.

Panel A shows homologies related to the 31-basepair consensus α2 site that are located further upstream of the α2 site. Underlining in the consensus α2 binding site corresponds to perfectly conserved bases (see Figure 3-9). Double lines under bases means that those bases match the consensus sequence. Numbers at right indicate the distance in nucleotides to the ATG of the relevant gene.

Panel B shows that the 31-basepair α2-binding site is quite homologous to a sequence identified by Siliciano and Tatchell (62) as necessary for a1-α2 regulation of the MATα1 gene: their deletion number R14 removes the sequences indicated by heavy underlining and results in constitutive expression of MATα1. Vertical lines indicate homology. Underlining in the consensus α2-binding site corresponds to perfectly conserved bases, as above.

Panel C shows homologies between the non-A/T-rich portion of the potential STE6 UAS region, and other upstream parts of the BAR1 and MFA1 genes. Underlining indicates sequences that match the STE6 "UAS". The STE6 deletion analysis indicated that sequences between d61 and d53 are required for orientation independent expression, and potentially contain a UAS. While gazing for sequence homology I decided to ignore the A/T rich part of the d61 to d53 interval: all that remained are the sequences listed for convenience as the STE6 "UAS" region. The "UAS" sequence includes some nucleotides to the left of deletion d61.

FIGURE 3-10. DNA sequence homologies

A. Upstream homologies related to the consensus α2-binding site.

		Distance upstream of α2 site
CONSENSUS α2 BINDING SITE	CATGTAATTACCNAA[T/A]AAGGAAATTTACATG	
MFA1	ATGTAATCAACTACTTTTTATTTTCTATGT^{-337}	103 bp
STE6	GCTGTTATTACCTACTAGTAATATCCTCTCT^{-351}	139 bp
BAR1	GGAAAGGCTCATAAAGGAAATTCAGGCT^{-438}	176 bp
STE6	ATTCCCACAGAGGGCATTTGAAATTTTATTT^{-425}	213 bp (=putative UAS)

B. Consensus α2-binding site vs a site required for a1-α2 repression.

CONSENSUS α2 BINDING SITE	CATGTAATTACCNAA[T/A]AAGGAAATTTACATG
SITE REQUIRED FOR 1-α2 REGULATION OF MATα1 (62)	TTTATTGCTTCCCAATGTAGAAAAGTACATC

C. Putative STE6 UAS region vs 5' flanking regions of BAR1 and MFa1.

		Approx. distance from ATG
STE6 "UAS" REGION	ACAGAGGGCATTTGAAATTTTAT	-230
MFA1	ACAGA GGCATTTATGGGCTTAG	-210
BAR1	ATTTGTTGCATTTATTATATTAG	-60
BAR1	ACAGATTTGATGTGGAAGATGAA	-340
MFA1	CAACGTGGCATAAGCTATGTAAT	-140

CHAPTER 4

THE IDENTIFICATION AND ANALYSIS OF *a*-SPECIFIC STERILE (NONMATING) MUTANTS OF YEAST

INTRODUCTION

The two haploid cell types of the yeast Saccharomyces cerevisiae, a cells and α cells, are specialized to mate and form the third cell type, the a/α diploid. Conjugation is a complex process that requires the function of gene products uniquely expressed by each of the haploid cell types. In particular, the distinctive sex pheromones secreted by a cells (a-factor) and α cells (α-factor) play central roles in conjugation: each pheromone induces in the opposite cell type changes that are critical for mating (reviewed by THORNER 1982; and SPRAGUE, BLAIR and THORNER 1983). For example, exposure of a cells to α-factor causes cell cycle arrest at the G1 stage ("start") and induces the expression or activity of several gene products (MANNEY 1983; STRAZDIS and MACKAY 1983) including cell-surface agglutinins responsible for adhesion to α cells. a-factor has symmetric effects on α cells, in synchronizing the cell cycle and inducing expression of gene products required to continue and complete conjugation (HAGEN and SPRAGUE 1984).

Analysis of mutants defective in mating ("sterile" mutants) has identified three functional classes. The "nonspecific" STE genes are required for mating proficiency by both a and α cells. In contrast the "α-specific" STE genes are required only by α cells for mating; there is no mating defect when an α-specific ste mutation is carried by an a cell. Likewise, the "a-specific" STE genes are required only by a cells for mating ability and have no phenotype in an α cell. Many but not all a- and α-specific STE genes are under transcriptional regulation by the mating type locus, MAT (reviewed by SPRAGUE, BLAIR and THORNER 1983).

The a-specific and α-specific STE genes encode products relevant to cell biology, including hormone receptors, peptide hormones, and enzymes that process the precursors of peptide hormones. For instance, the STE2 gene has been shown to code for the α-factor receptor (JENNESS, BURKHOLDER and HARTWELL 1983), and the α-specific STE3 gene may encode a component of the corresponding receptor for a-factor (HAGEN and SPRAGUE 1984). STE6 and STE14 are required for a-factor biosynthesis but their functions are not known (CHAN et al 1983; HAGEN and SPRAGUE 1984; WILSON 1984). The processing of the α-factor precursor encoded by the MFα1 gene (KURJAN and HERSKOWITZ 1982) has been elegantly defined at the biochemical level using α-specific ste mutants defective in α-factor biosynthesis (JULIUS et al. 1983; JULIUS, SCHEKMAN and THORNER 1984; JULIUS et al. 1984).

Genes required specifically for a cell fertility have been identified one at a time, by a variety of methods. Selective conditions imposed in the first ste mutant hunts required that cells be resistant to cell cycle arrest by pheromone, yielding many nonspecific ste mutants but only one a-specific (ste2) mutant (MACKAY and MANNEY 1974a,b; MANNEY and WOODS 1976; HARTWELL 1980). Such selections necessarily precluded finding sterile mutants that responded normally to α-factor. Indeed, the α-factor-sensitive ste6 and ste14 mutants were identified by nonselective methods: STE6 was identified in a screen for sterile a cells (RINE 1979), and both STE6 and STE14 were identified as "mex" mutations that prevented diploidization of homothallic strains-- spores grew into colonies composed of fertile α cells and sterile a cells (STE14: BLAIR 1979; MEX1 and MEX2: OSHIMA and TAKANO 1980). We have shown that mex1 and mex2 are alleles of ste6 and

ste14, respectively (see Appendix 1).

Some genes have been identified that encode products made only in a cells, but these genes have not been identified genetically as a-specific ste mutations for different reasons. The BAR1 gene (SPRAGUE and HERSKOWITZ 1981; CHAN and OTTE 1982) is expressed only in a cells but would not be found in ste mutant hunts because BAR1 function is not required for a cell fertility. There are two genes encoding a-factor, called MFA1 and MFA2, that were isolated by recombinant DNA techniques (BRAKE et al. 1985) based on the amino acid sequence of a-factor (BETZ, MANNEY and DUNTZE 1981). Although a-factor is undoubtedly required for a cell fertility, it is not yet known if mutation of a single a-factor structural gene would result in a sterile phenotype.

To further understand the physiology of mating we wanted to systematically identify all the a-specific ste genes, to determine if there were such genes that had not been identified in previous mutant hunts. A systematic approach had to deal with the following facts. We expected that the majority of sterile mutants identified would fall into the nonspecific class: such mutations cause sterility in both a cells and α cells. To determine which class each mutant belonged to would require that each be mated; the resulting diploid would then have to sporulated and dissected, and the mating phenotype of individual spores determined. Sterile mutants are by definition defective in mating (although rare mating events can be recovered), and dissecting on such a large scale as a screening method would be tedious.

Our approach was to design a strain that simplified the process of distinguishing a-specific from nonspecific ste mutants, and new from old STE complementation groups. We describe a mutant hunt using a

strain that is phenotypically an *a* cell at 25° but becomes an α cell at 34°. Thus no crosses were required to distinguish *a*-specific from nonspecific *ste* mutants. Our mutants comprise alleles of the previously-identified *a*-specific genes *STE2*, *STE6*, and *STE14*, and at least one new complementation group that we call *STE16*. The phenotype of *ste16* mutants indicates that *STE16* (in addition to *STE6* and *STE14*) is required for *a*-factor biosynthesis.

METHODS

Strains, genetic methods, and media: Yeast strains used are listed in Table 4-1, and crosses are listed in Table 4-2. Unless otherwise noted, all strains were constructed during this work. Crosses, sporulation, and tetrad dissection were performed as described previously (SPRAGUE and HERSKOWITZ 1981). Matings involving mating-deficient strains were performed by selection for prototrophy. Mating of sterile mutants derived from strain K12-14b was facilitated by pregrowth at 34°, where the mutants are fertile α cells.

Yeast rich medium (YEPD) and minimal medium (SD) were prepared as described previously (HICKS and HERSKOWITZ 1976).

Assays of mating phenotype: Mating phenotype was assayed on plates as described (WILSON and HERSKOWITZ 1984) using strains 70, 227, and 6B78 for mating type lawns, strain RC757 for detecting a-factor, and strain XMB4-12b for α-factor detection.

Construction of strain K12-14b: The parent strain for the mutant hunt, K12-14b, was constructed from segregants of crosses K10, K11, K17, and K12. The salient features of K12-14b (*mata HMLα HMRα sir3-8^{ts}*) are described fully in Results. The presence of the *rme1* allele in the parent strain was critical for subsequent genetic analysis of the mutants, since the *rme1* mutation allows cells lacking *MATa* information to sporulate (albeit poorly) (KASSIR and SIMCHEN 1975; RINE, SPRAGUE and HERSKOWITZ 1981). The *rme1* mutation in *mata* strains was followed by crossing with *rme1* tester strains such as 184A-2b and 866; such diploids sporulate only if homozygous for *rme*. We noticed that diploids heterozygous for the *sir3-8* mutation frequently became *sir3-8* homozygotes during mitotic growth (K. Wilson, data not shown), perhaps

as a consequence of mitotic recombination within the ribosomal RNA gene cluster centromere-proximal to SIR3 on chromosome XII (BROACH 1982; MORTIMER and SCHILD 1982). sir mutants express mating type information from the silent loci HML and HMR and can thus sporulate if wildtype a and α information is present at HML or HMR. To determine unambiguously the presence of the rme1 allele, strain K12-14b (mata sir3-8^{ts}) was mated with strains carrying only α information (strains 184A-2b and 184A-15c). Because K12-14b itself carried no wildtype a information the resulting diploids (not listed in Table 4-2) were devoid of wildtype a information. Therefore, the sporulation seen was indeed due to homozygosity of the rme1 mutation, and strain K12-14b was thereby determined to carry the rme1 mutation. Spores from these diploids were dissected to verify that strain K12-14b, when crossed, exhibited normal spore viability and 2:2 segregation for the expected markers (data not shown). Strain K12-14b may be transformed with plasmid DNA using the glusulase method (BEGGS 1978).

Mutagenesis of strain K12-14b: The parent strain K12-14b was mutagenized by exposure to ethylmethane sulfonate (EMS; Eastman Kodak Co.) as described (OSHIMA and TAKANO 1980), except that cells were exposed to EMS for one hour. To assure independent isolation of mutants, ten single colonies of K12-14b were treated independently, forming mutagenesis series A through J. The frequency of survivors following the one-hour exposure to EMS was approximately 4%. The effectiveness of mutagenesis was estimated by plating out and screening colonies for resistance to canavanine (the parent strain carried the canavanine-sensitive CAN1 allele) and by a red to white color assay: the parent colonies are red, due to the ade2-1 mutation, and a color

change from red to white may be due to mutations at ade4, ade5, ade6, ade7 or ade8 (JONES and FINK 1982) or to tRNA suppressor mutations (ade2-1 is an ochre-suppressible allele). The frequency of mutation of a given locus estimated by these two methods was a maximum of 2%.

RESULTS

Mutant isolation strategy: the phenotypes of starting strain K12-14b: We wanted to identify additional loci required for the *a* mating phenotype. The class of mutants we sought was by definition mating-deficient. We therefore constructed a strain that would simplify mutant analysis in the following two ways. First, we needed a method other than mating, sporulation, and tetrad dissection to distinguish recessive *a*-specific sterile mutations from non-specific sterile mutations, since in previous mutant analyses (MACKAY and MANNEY 1974a,b; MANNEY and WOODS 1976; HARTWELL 1980) the non-specific class constituted the majority of the mating-deficient mutants isolated. Second, we needed an easy method to distinguish mutations in new complementation groups from mutations in the previously identified *a*-specific genes *STE2*, *STE6*, and *STE14*. The strain we constructed to accomplish these goals is named K12-14b.

Strain K12-14b was designed so that its mating phenotype is controlled by temperature: at low temperature the cells mate as *a*, and at high temperature the cells mate as α, as shown in Figure 4-1. The temperature sensitivity of the mating phenotype is due to a temperature-sensitive allele of *SIR3*, *sir3-8*^{ts}, identified by J. Rine (RINE 1979). The *SIR* gene products inhibit transcription of the two silent copies of mating type information located at the left (*HML*) and right (*HMR*) ends of chromosome III (reviewed by HERSKOWITZ and OSHIMA 1982; NASMYTH 1982). *S. cerevisiae* normally carries α information at *HML* (*HMLα*) and *a* information at *HMR* (*HMRa*). Strain K12-14b in contrast carries α information at both silent loci. Furthermore, K12-14b carries the recessive *mata* allele. At low temperature (25°) the *sir3-8*

gene product represses transcription of HML and HMR, and the cells are phenotypically a (no mating type information is necessary to establish the a phenotype). However, at high temperature (34°) the sir3-8 gene product no longer represses the silent loci, and α information is expressed (see Figure 4-1). K12-14b is defective in a1 activity due to the mata mutation, and thus at high temperature (30° or more) behaves as an α cell. (If the strain had carried a wildtype MATa allele it would have behaved as a diploid (nonmater) at high temperature because of a1-α2 activity.)

The mating behavior of K12-14b at intermediate temperatures is consistent with a gradual loss of sir3-8 activity as cells are grown at higher temperatures, whereby some expression from silent loci occurs but not full derepression. For example, colonies pregrown and tested for mating phenotype at temperatures between 25° and 30° mate both as a and as α, and produce both a-factor and α-factor. Figure 1 shows that even at 25° some cells in the colony mate as α. However, this bisexual phenotype has two possible origins: single cells could be either a or α in phenotype, or single cells could express both phenotypes simultaneously as occurs in matα2 mutants (STRATHERN, HICKS and HERSKOWTIZ 1981). Cells of strain K12-14b grown at 25° and examined under the microscope exhibit the "shmoo" morphology typical of both matα2 cells and single cells of either mating type after prolonged exposure to the mating pheromone of the opposite sex.

The utility of the design of K12-14b is that mutants derived from it may be tested for their ability to mate as a and their ability to mate as α simply by pregrowing the cells at low or high temperature and testing with the appropriate mating type tester lawn. Thus no crosses

or dissection are necessary to distinguish a-specific from non-specific STE mutations. Furthermore, complementation testing that requires mating with sterile a tester strains is facilitated by pregrowing the mutants at high temperature so that they mate as α cells: rare matings between a cells and α cells with ste mutations are more frequently obtained than rare matings between two a cells carrying ste mutations (RINE 1979).

Mutagenesis of K12-14b and initial screening of mutants: Strain K12-14b was mutagenized using EMS to approximately 4% survival. Ten independent mutagenesis series, called A through J, were performed to assure independence of isolation. Mutagenized cells were plated on YEPD and grown to single colonies (approximately 400 colonies per plate, ten plates per mutagenesis series) at low temperature (25°). This "master" plate was then replica plated to two YEPD plates that were grown overnight, one at 25° and the other at 34°. The colonies grown at 25° (where the parent mates as a) were tested for their ability to mate as a by replica plating each plate to a lawn of α cells (strain 70) on minimal medium (SD plates). Mating yields diploids that are prototrophic and grow on minimal medium. Colonies unable to mate as a were visualized as "ghost" colonies. Likewise, the colonies pregrown at 34° (where the parent mates as α) were tested for their ability to mate as α by replica plating to a lawn of a cells (strain 227). The pairs of mating tester plates were then compared side-by-side. We looked for colonies that did not mate as a (ghost colonies at low temperature) but did mate as α (prototrophic colonies at high temperature). Over one hundred mutant colonies identified from this initial screen were found on the YEPD master plates and streaked onto

YEPD to isolate and retest single colonies for mating phenotype. Seventy-two mutants retested as potential a-specific sterile mutants.

In addition to potential a-specific mutants we picked a small sample of sterile mutants defective for mating at both temperatures. We chose 62 mutants at random from the large number of nonspecific sterile mutants present on the screening plates, for future genetic and phenotypic analysis.

Screening out mutants defective in functions other than ste gene function: Strain K12-14b carries a temperature-sensitive sir3 allele which determines its different mating properties at different temperatures. However, acquisition of a nonconditional mutation at any SIR locus would result in expression of mating type information from the silent copies at all temperatures; such cells would mate as α at all temperatures. Because there are six different SIR genes (KASSIR and SIMCHEN 1985; RINE and HERSKOWITZ 1985; G. SIMCHEN, pers. comm.), a large proportion of the mutants were expected to fall within this class. Indeed, 55% of the mutants obtained (39 out of 72) mated as α at low and at high temperature; these were assumed to be sir mutants and were not studied further.

The α strain (70) used to test for mating ability is a thr3 auxotroph. Mutants defective in thr3 gene function are unable to form prototrophs in the mating test and falsely appear to be mating deficient. Five mutants were unable to grow on medium lacking threonine and were eliminated from further consideration.

Table 4-3 shows the number of mutants remaining after each screening step was performed. In the end, 28 potential a-specific ste mutants remained.

Complementation analysis with a-specific STE mutants: The 28 mutants were assigned to complementation groups based on their ability to complement the mating defect of a strains carrying mutations in the known a-specific STE genes STE2, STE6, and STE14. Mutants were pregrown at 34° to make them phenotypically α, and then mixed with each of three tester strains. The tester strains were MATa and carried a mutation in either ste2, ste6, or ste14 (strains 189, K39-3b or K39B-12c, and HR129-5d, respectively). The strains were mixed, allowed to grow and mate on rich plates for 5-12 hours, and replica plated to minimal medium (supplemented with shared nutritional requirements) to select diploids by complementing auxotrophic markers. In each case the MATa/mata diploid so formed was homozygous for at least one auxotrophic marker, allowing its mating phenotype to be determined by replica plating to a and α tester lawns on minimal medium. If the mutant was defective in a ste gene different from the ste gene of the tester strain, then the a/a- diploid was able to mate as a, and the mutant was said to complement that tester strain. Mutants were assigned to a known complementation group (STE2, STE6, or STE14) if they formed non-mating a/a- diploids with one of the testers. The complementation data are presented in Table 4-4 and tabulated in Table 4-7. Most of the mutants were assigned to a known complementation group: we identified thirteen presumptive ste6 alleles (A66, B28, B60, B61, C1, C2, C11, D11, E3, E5, F20, H7, I2), four presumptive ste14 alleles (B36, B39, C8, J3), and two presumptive ste2 alleles (B64, D25). Complementation analysis of seven mutants has not been completed (A20, D7, D15, D29, E9, G6, and H5).

Testing for possible dominant ste mutations: A mutant that failed to complement one tester strain and did complement the other two tester strains was assumed to be a recessive allele of the first strain. A mutant failed to complement more than one tester strain was considered a potential dominant mutation. Mutant G6 failed to complement, or only partially complemented, all three ste tester strains. Outcrossing of the G6 mutation into a normal genetic background will be necessary to investigate its phenotype further.

Identification of mutations in at least one new complementation group: Most of the mutants isolated are alleles of STE2, STE6, and STE14, based on their behavior in complementation tests. Mutants I11 and I14, however, were able to complement the mating defects of all three tester strains and therefore define at least one new complementation group. Mutants I11 and I14 were both derived from mutagenesis series I and are therefore not necessarily independent mutations. We believe that they are independent mutations for three reasons. First, they are both deficient in a-factor production as assayed by the halo assay, but I14 appears more deficient than I11. Second, mutant I14 grows poorly at low temperature and is temperature-sensitive for growth (consequently, its mating phenotype is difficult to assay at temperatures above 30°), while the I11 mutant grows normally. Third, diploids formed with the I14 strain fail to sporulate, while diploids formed with mutant I11 sporulated adequately. Complementation analysis is in progress to determine whether the I11 and I14 mutations represent the same or different complementation groups.

The Ill mutation segregates as an a-specific sterile mutation: Further analysis of the Ill mutation required crossing it into conventional genetic backgrounds. We rare-mated mutant Ill to a *MATa rme* strain (JM153A-6c) by selection for prototrophy on minimal medium to form diploid K43. Sporulation and dissection of K43 yielded segregant K43-4c. In general, diploids from the first *rme*-dependent (see Methods) mutant outcross sporulated quite poorly, probably because the mutants had been so heavily mutagenized. Subsequent crosses sporulated better, and served to separate the *ste* mutation from undesired mutations including the parental *mata* and *sir3-8* markers. Strain K43-4c was mated with strain 381G to form diploid K50. Meiotic products of diploid K50 were used for genetic analysis of the Ill mutation, and to construct a strain suitable for cloning the *STE16* gene by complementation (strain K91-3b). As shown in Table 4-5, tetrads from two crosses demonstrate that the Ill mutation caused mating deficiency only in *a* cells: there were two fertile α spores in every tetrad. The Ill mutation and the mating type locus are unlinked: half of the *MATa* spores were mating proficient and half were mating defective. The phenotype of the Ill mutation does not depend on the recessive *mata* allele, because segregant K43-4c carries the wildtype *MATa* allele of parent strain JM153A-6c and still exhibits the *a*-specific mating defect of the original mutant. (The *MATa* allele was identified by cosegregation with the cryptopleurine resistance marker *cryl*.)

The Ill mutation is not allelic with ste2, ste6, or ste14, and therefore defines a new locus required for fertility of MATa *cells*: If the Ill mutation indeed represents a new locus required for *a* cell fertility, it should be genetically separable from the three known *a*-

specific STE genes. The crosses shown in Table 4-6 demonstrate that the ill mutation is distinct from STE2, STE6, and STE14, because in each cross recombination during meiosis yielded fertile a spores. To test the possibility that the fertile a segregants of crosses K85 and K87 represented the segregation of an unlinked suppresor of the mating deficiency, a fertile a segregant from each cross was mated with the fertile α strain 1369, sporulated, and dissected (Table 4-6); no sterile a spores were found among the meiotic products of crosses K89 and K93, indicating that an unlinked suppressor was not present.

We suggest that the ill mutation represents a new complementation group required for mating proficiency in a cells, but not α cells. We designate the new locus STE16, and the ill allele ste16-1.

The phenotype of MATa ste16-1 mutants: In addition to the inability to mate efficiently, MATa strains carrying the ste16-1 mutation are defective in the biosynthesis of a-factor pheromone, shown in Figure 4-2. In segregants of crosses K50 and K91 (Table 4-6) the mating defect cosegregated with a defect in a-factor production: all 14 sterile a segregants were a-factor deficient and all 12 fertile a segregants displayed wildtype a-factor activity. The ste16-1 mutation may be leaky, since a strains carrying the mutation are able to mate at low frequency. Other phenotypes related to mating, such as sensitivity to cell cycle arrest by α-factor, agglutination with α cells, and production of the Barrier activity that degrades α-factor, have not yet been investigated. However, deficiency in a-factor production would be sufficient to cause the observed sterile phenotype of a ste16-1 cells.

DISCUSSION

We have isolated a number of a-specific ste mutants defective in mating. We recovered two or more independent alleles of each of the previously known a-specific genes STE2, STE6, and STE14. In addition, we have identified at least one new complementation group, called STE16, and showed that it is not allelic with the other a-specific STE genes. When diploids heterozygous for ste16 are sporulated and dissected, the a-mating deficiency cosegregates with a defect in a-factor production. As shown in Figure 4-2, the STE16 gene product thus joins the STE6 and STE14 gene products in being required for some aspect of a-factor pheromone processing or secretion. (Another possibility is that STE16 is required for a-factor gene expression). We have recently isolated a DNA fragment containing the STE16 gene by complementation of the mating defect of the a ste16 strain K91-3b (P. Garcia and K. Wilson; see Appendix 4). Comparison of the restriction map of this cloned DNA with those of two a-factor structural genes indicates that STE16 is probably not an a-factor structural gene (BRAKE et al. 1985).

The ste16 mutant we identified will be useful in studying pheromone biosynthesis in a cells. It seems clear that the a-factor and α-factor precursors are processed differently. Mutations in two genes, ste13 and kex2, whose products perform specific proteolytic cleavages of the α-factor precursor (JULIUS et al. 1983; JULIUS et al. 1984) have no phenotype in a cells and thus these genes are not required for a-factor processing. Conversely, we now know of three genes, STE6, STE14, and STE16, that are required for a-factor biosynthesis and that have no phenotype in α cells. The structure of

the a-factor precursors predicted by their DNA sequences indicates that a minimum of one step (separating the mature pheromone from the C-terminal precursor polypeptide) is necessary to liberate mature a-factor (BRAKE et al. 1985). However, mature a-factor pheromone may be a 15-mer peptide and not an 11-mer as initially reported, because chemically synthesized 11-mer peptide is biologically inactive (J. BECKER, pers. comm.). A report of covalent attachment of farnesyl moieties (a fatty acid) to carboxyterminal cysteine residues in several species of Basidiomycetes yeast (SAKAGAMI et al. 1981) has prompted speculation that a-factor may be similarly modified (BRAKE et al. The functions performed by the STE6, STE14, and STE16 gene products do not overlap in the sense that overexpression of STE6 or STE14 on high copy number plasmids does not suppress the mating defect of a ste16 strain. Likewise the ste6 defect is not suppressed by overexpression of STE14 and the ste14 defect is not suppressed by overexpression of STE6 (see Appendix 4).

It is interesting that the two genes required to process α-factor are expressed and their gene products are active in all three yeast cell types (SPRAGUE, BLAIR and THORNER 1983). In contrast, STE6 is regulated by the α2 product of the mating type locus: the STE6 gene is transcribed only in a cells (WILSON and HERSKOWITZ 1984), and so the STE6 product may be involved exclusively with a-specific gene products or even exclusively with a-factor. We do not yet know if STE14 or STE16 are similarly regulated by the mating type locus.

Our approach to isolating a-specific ste mutants was successful and resulted in the identification of one new complementation group (STE16) in addition to the three known ones. Further genetic and

phenotypic analysis of the incompletely-characterized mutants is necessary to determine if we isolated any additional mutant classes. A particularly interesting class of mutants that we failed to identify are those defective in agglutination. If mutants D7 and H5, which have wildtype a-factor activity, complement a ste2 tester strain upon further testing, they would be candidates for agglutination or other (non-a-factor-related) defects. Strain backgrounds vary considerably with respect to agglutination properties, and we do not know if our parent strain was suitable for detecting agglutination defects.

Are there other a-specific STE genes? It may be that mutants defective in STE2, STE6, STE14, and STE16 comprise the single-copy nonessential a-specific STE genes, and that other loci exist that have not yet succumbed to genetic identification. For example, where two genes code for similar gene products, as is the case for the two a-factor genes, a single mutation may not result in a sterile phenotype. To get a quantitative feeling for the probability of having missed an a-specific STE gene in our mutant hunt, we performed the following calculation. If we assume that there are five a-specific STE genes, then the probability of having found no mutants in one class can be calculated from the Poisson distribution of the number of independent mutants actually obtained in each of the four complementation groups (Table 4-7). This probability is $P(0) = e^{-n}$, where n is the average number of independent mutants found for each STE gene. If $n = 14/5 = 2.8$, then $P(0) = 0.06081$. There is thus a 6% chance that we failed to identify any representatives of an unknown single-copy a-specific STE gene in this mutant hunt.

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TABLE 4-1. Strain list

NAME	GENOTYPE	SOURCE*
6B78	MATa ade5 his5 ura4 met4 met13 HO^-	L. Blair
70	MATα thr3-10	F. Sherman
184A-2b	MATα hmrα2 rme1 his3 trp5 ura3 leu2 can1 cyh2 met MAL2	J. Margolskee
184A-15c	MATα hmrα2 rme1 his3 ura1 leu2 ade2 can1 cyh2 met MAL2	J. Margolskee
189	MATa cryR (selected by KLW) ste2-1 his2 ade2-1-oc lys1-1 trp5-18 gal2 can1	T. Manney
227	MATa lys1-1 cryR	J. Hicks
381G	MATα cryR ade2-1-oc his4-580-am lys2-oc trp1-am tyr1-oc SUP4-3-ts-am	L. Hartwell
763	MATa ste6-3 (RSA3 allele) ade6 lys2 arg4	J. Rine
854	MATα sir3-8-ts his4-580-am ade2-1-oc lys2-oc tyr1-oc trp1-am met	J. Rine
866	mata leu1 ade6 trp5 ura3 can^r rme	G. Sprague
947	MATα ste2-1 ade2-1 lys1-1 hom3 iso1 can1 (his2?)	L. Hartwell
1369	MATα isogenic with HR125-5d	R. Jensen
HR101A-E1	MATa cryR rme his4-am trp1-am lys2-oc tyr1-oc ura3	R. Jensen
HR125-5d	MATa leu2-3 leu2-112 ura3-52 trp1-am his3 his4	R. Jensen
HR125-11b	MATα ura3-52 leu2-3 leu2-112 his3 his4 trp1-am	R. Jensen
HR129-2d	MATa ste14-1 cryR (selected by KLW) leu2-3 leu2-112 ade5 canR cyhR	R. Jensen
HR129-5d	MATα cryR (selected by KLW) ste14 leu2-3 leu2-112 ura4 met his3 and/or his5	R. Jensen
JM153A-6c	MATa cryR rme lys2 ura3	J. Margolskee
K10-17c	MATα rme sir3-8-ts ade2-1-oc his4-580-am trp1-am lys2-oc tyr1-oc met	
K11-23b	mata HMLα HMRα rme ade2 his4-am lys2-oc trp1-am tyr1-oc met	
K12-14b	mata HMLα HMRα sir3-8-ts rme leu2-3 leu2-112 ura3-52 ade2-oc lys2-oc trp1-am his4-am his3	

(continued on next page)

TABLE 4-1, continued.

NAME	GENOTYPE	SOURCE*
K17-8a	mata HMLα HMRα rme sir3-8-ts ade2-oc his4-580-am lys2-oc trp1-am tyr1-oc met	
K39-3b	MATa ste6-21 leu2-3 leu2-112 his4 ade6 lys2 can1	
K39B-12c	MATa ste6-21 arg4 his4 leu2-3 leu2-112 lys2	
K43-4c	MATa cryR ste16-1 (111 mutation) leu2-3 leu2-112 ura3 trp1-am tyr1-oc his3 his4 met	
K48-4b	MATα ste14-2 (mex2 allele of TAKANO) ura3-52 ade2-1-oc leu2-3 leu2-112 his4 trp1-am SUP4-3-ts-am (his3?)	
K50-1a	MATa cryR ste16-1 trp1-am ade2-1-oc lys2-oc ura3 tyr1 SUP4-3-ts-am his3 and/or his4-am	
K50-1b	MATa cryR ste16-1 ade2-oc leu2-3 leu2-112 lys2-oc tyr1-oc trp1-am his4-am	
K50-5c	MATα cryR ste16-1 ade2 leu2-3 leu2-112 tyr1-oc trp1-am his4-am SUP4-3-ts-am	
K77	MATa ste6::lacZ (isogenic with HR125-5d)	
K85-1b	MATa leu2-3 leu2-112 tyr1-oc lys2 (fertile)	
K87-3c	MATa cryR ade2 lys2-oc tyr1-oc leu2-3 leu2-112 met (fertile)	
K91-3b	MATa cryR ste16-1 ura3-52 leu2-3 leu2-112 lys2-oc tyr1-oc trp1-am his4	
RC757	MATα sst2-1 met1 his6 can1 cyh2	R. Chan
XMB4-12b	MATa sst1-1 ilv3 arg9 ural killer$^+$	L. Blair
XR160-12b	mata HMLα HMRα rme1 ade2 ura3 leu1 can1-11 cyh2-21	J. Rine

*Strains constructed by K.L. Wilson unless otherwise noted.

TABLE 4-2. Crosses

Cross	Parents
K10	854 X R101A-E1
K11	K10-17c X XR160-12b
K12	K17-8a X HR125-11b
K17	K11-23b X K10-17c
K43	I11 mutant X JM153A-6c
K50	K43-4c X 381G
K52	K50-1a X K48-4b
K83	K50-5c X 189
K85	K50-5c X 763
K87	K50-1b X HR129-5d
K89	K87-3c X 1369
K91	K50-1b X 1369
K93	K85-1b X 1369

TABLE 4-3. Initial mutant screening

Mutagenesis series	Potential *a*-specific mutants*	Presumptive *sir* mutants**	*thr* mutants	Remaining mutants
A	10	7	0	3
B	13	7	0	6
C	7	2	1	4
D	14	9	0	5
E	7	3	1	3
F	3	2	0	1
G	5	3	1	1
H	3	2	0	1
I	4	1	0	3
J	6	3	2	1
TOTALS	72	39	5	28

*Colonies that did not mate with an α lawn at 25° but did mate with an *a* lawn at 34°.

**Colonies that mated with an *a* lawn at 25°.

Legend to Table 4-4. a-factor production was determined by halo assay, where the distance from the edge of the colony or patch to the lawn defines the halo (see Figure 4-1). + indicates wildtype halo size; - indicates no halo; and a number such as 0.2 indicates a smaller than wildtype halo (in this example, the mutant halo size is 20% of wildtype).

nt means not tested; growth^{ts} indicates that the mutant strain does not grow at 34^{o} but does grow at lower temperatures; and leaky means that the mutant was able to mate (inefficiently) as a.

Complementation tester strains were 189 (ste2), K39-3b and K39B-12c (ste6), and HR129-2d (ste14). + indicates that the a/a^{-} diploid formed was able to mate as a with mating type tester strain 70; - indicates that the diploid was unable to mate as a; -/+ indicates that the diploid mated poorly as a.

TABLE 4-4. Mutant a-factor phenotypes and complementation analysis

Mutant	a-factor phenotype	Complementation with ste2$^-$	ste6$^-$	ste14$^-$	Remarks
A66	0.2	+	-	+	
B28	0.2	nt	-	+	
B60	0.3	+	-	+	
B61	0.1	+	-	+	
C1	0.2	nt	-	+	growth^{ts}
C2	0.3	nt	-	+	growth^{ts}
C11	0.3	+	-	+	
D11	0.2	+	-	+	
D15	0.2	+	nt	+	
E3	0.2	+	-	+	
E5	0.2	+	-	+	
F20	0.2	+	-	+	
H7	0.2	+	-	+	growth^{ts}
I2	0.3	+	-	+	
B36	-	+	+	-	
B39	-	+	+	-	
C8	-	nt	+	-	
J3	-	nt	+	-	growth^{ts}
B64	+	-	+	+	
D7	+	nt	+	+	
D25	+	-	+	+	leaky
E9	+	nt	nt	+	
I11	0.1	nt	+	+	
I14	-	+	+	+	growth^{ts}
A20	+	nt	-	nt	growth^{ts}
D29	0.3	nt	-	nt	growth^{ts}
G6	0.5	-/+	-	-/+	growth^{ts}
H5	+	nt	nt	nt	

TABLE 4-5. *ste-I11* segregates as an *a*-specific *STE* gene.

Cross	Description	Number of tetrads		
		2 *a*: 2 α	1 *a*: 1 nm: 2 α	2 nm: 2 α
K50	*a ste-I11* X α *STE*	1	4	1
K91	*a ste-I11* X α *STE*	2	2	3

TABLE 4-6. *ste-I11* is not an allele of *STE6* or *STE14*.

Cross	Description	Number of tetrads		
		2 *a*: 2 α	1 *a*: 1 nm: 2 α	2 nm: 2 α
K85	α *ste-I11* X *a ste6*	0	4	3
K87	*a ste-I11* X α *ste14*	0	4	1
K52	*a ste-I11* X α *ste14*	0	3	3
K89*	*a STE* X α *STE*	8	0	0
K93**	*a STE* X α *STE*	7	0	0

* *a STE* strain is a fertile segregant from cross K87
** *a STE* strain is a fertile segregant from cross K85

TABLE 4-7. Number of mutants obtained in each complementation group.

Complementation Group	Mutant names	Number of independent mutants
STE6	A66	8
	B28, B60, B61	
	C1, C2, C11	
	D11	
	E3, E5	
	F20	
	H7	
	I2	
STE14	B36, B39	3
	C8	
	J3	
STE2	B64	2
	D25	
STE16	I14	1

Legend to Figure 4-1. At right, the mating phenotype of strain K12-14b at 25° and at 34° is shown. The upper patch on each plate is strain K12-14b, the lower right patch is a fertile a control (strain HR125-5d), and the lower left patch is a fertile α control (strain 1369). The three patches were grown overnight on YEPD and then replica plated to two separate YEPD plates: one was grown at 25° and the other at 34°. Each plate was then tested for mating ability and pheromone production by replica plating to the appropriate tester lawns as described in Methods. Strain K12-14b clearly has opposite mating ability at the two different temperatures; at 25° it mates efficiently as a (and less efficiently as α) and produces a-factor but not α-factor, while at 34° K12-14b mates only as an α and produces only α-factor.

The diagrams at left illustrates the effect of SIR function on expression of mating type information from the silent copies. Lines ending in a bar indicate inhibition of transcription; lines ending in an arrowhead indicate activation of transcription. The hollow bar represents chromosome III; the solid circle represents the centromere. Wavy lines represent transcription, and proteins are circled.

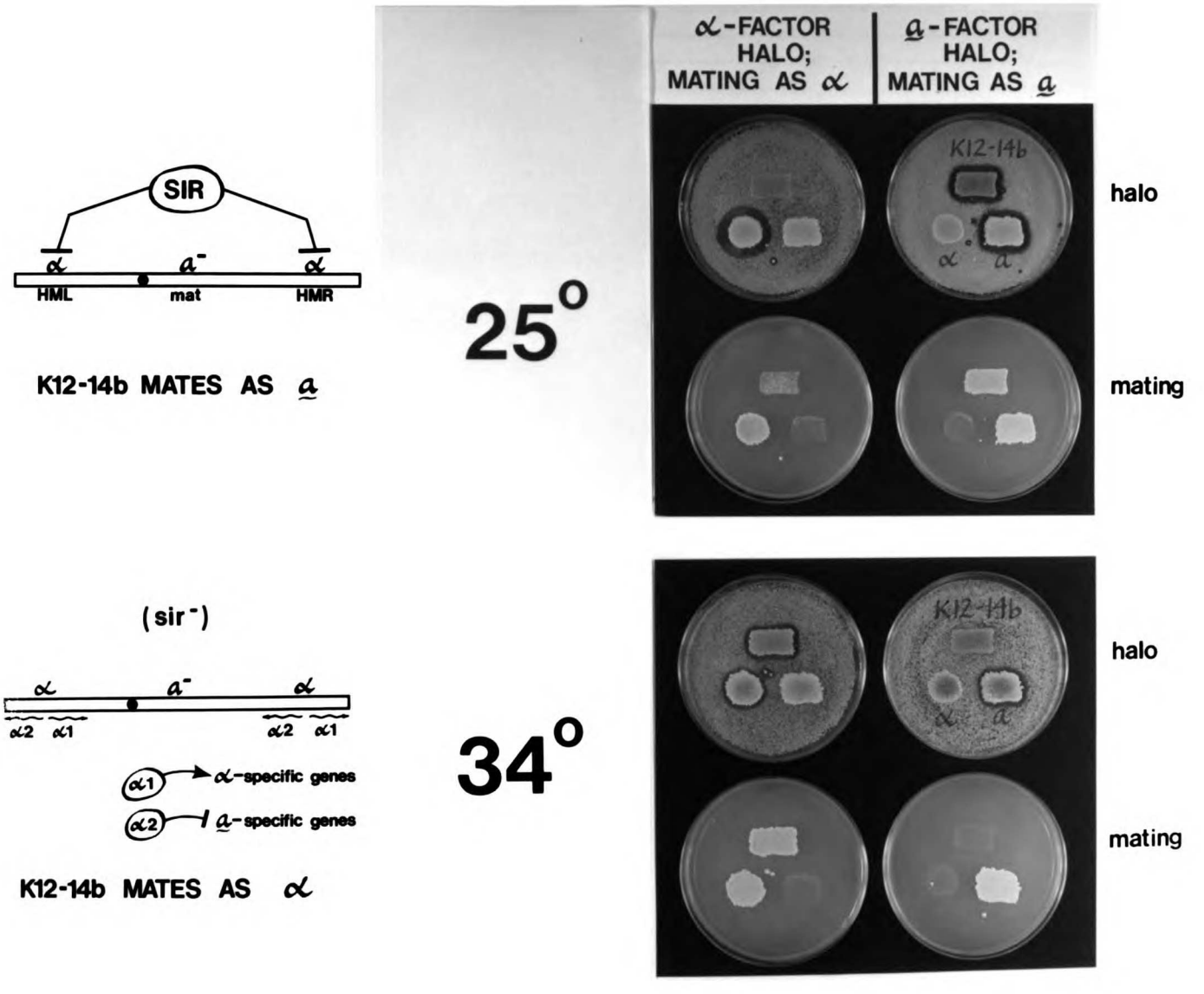
SIR
α
a⁻
α
HML
mat
HMR
K12-14b MATES AS a
(sir⁻)
α
a⁻
α
α2
α1
α2
α1
α1
α-specific genes
α2
a-specific genes
K12-14b MATES AS α
25°
34°
α-FACTOR HALO; MATING AS α
a-FACTOR HALO; MATING AS a
K12-14b
halo
mating
halo
mating

FIGURE 4-1

Legend to Figure 4-2. A side-by-side comparison of the a-factor deficiency phenotypes of a strains carrying mutations in ste6 (strain K77), ste14 (strain HR129-2d), and the newly identified ste16 (strain K91-3b). The positive control is a strain HR125-5d, and the negative control is α strain 1369. Plates were incubated at 25° to slow the growth of the halo tester lawn and maximize the sensitivity of the assay to low quantities (or aberrant forms of) a-factor produced by the mutant strains. Note that all three mutant strains produce a tiny but visible halo relative to the negative control.

FIGURE 4-2

APPENDIX 1

GENETIC ANALYSIS OF A TEMPERATURE-SENSITIVE STE6 MUTATION

INTRODUCTION

OSHIMA and TAKANO (1980) isolated mutants defective in diploidization of homothallic spores that carried mutations termed mex. Tetrad analysis was consistent with these being mutations in two genes (mex1 and mex2) required for fertility of a cells, and one gene (mex3) required for fertility in α cells. The mex1 allele was particularly interesting because its mating defect was manifested only at high temperature.

This appendix describes outcrosses to obtain the a-specific mex mutations in heterothallic strains, and allelism tests with the known a-specific genes STE2, STE6, and STE14. (The STE16 locus reported in Chapter 4 had not been identified at the time these crosses were done). Some work with the α-specific mex3 mutation is also described.

I show that mex1 is allelic with STE6, and that mex2 is allelic with STE14. Furthermore, the temperature-sensitivity of mating of the original mex1 mutant is associated with mex1 itself and is not due to an unlinked modifier present in the parent strain.

MATERIALS AND METHODS

Strains, media, and genetic methods: Yeast strains used are listed in Table 1A.1, and crosses are listed in Table 1A.2. Unless otherwise stated all strains were constructed for this work. Genetic methods were as described previously (HICKS and HERSKOWITZ 1976). All crosses including rare-matings with sterile strains were performed by selection for protrophy.

Assays of mating phenotype: Mating phenotype was assayed on plates as described (WILSON and HERSKOWITZ 1984) using strains 70, 227, 6B55, and 6B78 for mating type lawns, strain RC757 for detecting a-factor, and strain XMB4-12b for detecting α-factor.

Extracting the mex mutations from the HO background: The phenotypes of mutants 2-9 (mex1), 6-26 (mex2), and 5-17 (mex3) obtained from Oshima and Takano were as follows:

The mex1-ts strain at 35° was deduced by TAKANO and OSHIMA (1980) to be a mixture of sterile a cells and fertile α cells with a colony mating phenotype of α. At 25° the a cells regain fertility and mate with the α cells to form a diploid population capable of sporulation (the mex1 strain arrived in the mail as a diploid population).

mex2 colonies mated as α at all temperatures, being in theory a mixture of fertile α cells and sterile a cells.

mex3 colonies mated as a at all temperatures, being in theory a mixture of fertile a cells and sterile α cells.

To obtain the mutations in a heterothallic background (ho), mex2 α cells were mated with an a strain (HR125-5d) to form diploid K18, which was sporulated and dissected. Likewise, mex3 a cells were mated with an α strain (HR125-11b) to form diploid K19, which was dissected. The mex1 diploids were sporulated and dissected; the spores were germinated and grown at 34° (where the a cells are sterile) in which case the mex1 α cells could mate with a strain HR125-5d to form diploid K20. Spores from complete tetrads were scored for mating phenotype (at both low and high temperature for segregants of the mex1 cross), and the genotypes (HO vs ho, MATa vs MATα, and MEX vs mex) deduced directly from the

phenotype or inferred by assuming 2:2 segregation of the alleles. Outcrosses confirmed all genotype predictions (see below), and I identified an ho α spore carrying mex1 (strain K20-2d) and a ho α spore carrying mex2 (strain K18-6d). Strains K20-2d and K18-6d were crossed with strain HR125-5d (their second crosses into the HR125-5d genetic background) to form diploids K22 and K27, respectively. Sporulation and dissection of K22 and K27 resulted in the segregants used for allelism tests.

One complete tetrad analyzed from diploid K19 (the mex3 heterozygote) yielded spores K19-2c and K19-2d of predicted genotypes HO mex3 and ho MATa mex3; these spores had the same mating phenotype, and which spore had which genotype was not determined. One outcross not shown (only one tetrad) yielded a nonmating segregant that produced α-factor, suggesting that the mex3 mutation is different from KEX2 and STE13, genes required for α-factor biosynthesis (JULIUS et al. 1984). The putative mex3 phenotype is similar to that of ste3 mutants (HAGEN and SPRAGUE 1984).

RESULTS

The mex1 mutation is not allelic with ste2 or ste14: Two MATa strains carrying the temperature sensitive mex1 mutation were crossed under permissive conditions (25°) with α strains carrying either the ste2-1 or the ste14-1 mutation. As shown in Tables 1A.3 and 1A.4, all four crosses gave rise to mating-proficient a segregants, indicating that the mex1 mutation is not allelic with ste2 or ste14. In the mex1 by ste14 crosses K30 and K32, 29% of the MATa spores were able to mate; 25% of the a segregants were expected to be fertile if the two mutations are unlinked. However, only 12% of the MATa spores from

diploids K29 and K31 were mating-proficient, indicating that mex1 and ste2 may be linked genetically. STE2 is not centromere-linked, and maps distal to CDC4 on chromosome VI (A. Burkholder, D. Jenness, and L. Hartwell, personal communication).

The mex1 mutation is allelic with ste6: Strain K22-4d was an α segregant of a tetrad with 2 fertile a spores and 2 fertile α spores and was therefore predicted to carry the mex1 mutation. K22-4d was crossed with the original ste6 strain, namely the RSA21 isolate of RINE (1979), to form diploids K21A, K21B, and K21C (three independent colonies of RSA21 used as parents). RSA21 appears to contain several mutations, since when it is mated the resulting diploid sporulates poorly, rarely generating more than two live spores per tetrad. Furthermore, ste6 strains are leaky, in that some mating is detectable by the plate patch test, and a-factor "halos" can be up to half the halo size of wildtype a strains. As shown in Table 1A.5, a total of 58 MATa spores were assayed, of which 56 showed both mating and a-factor defects at 34°. Two spores showed near normal mating (by the patch test) but were still deficient in a-factor production. I tentatively conclude that mex1 and ste6 are allelic. This conclusion should be confirmed by a cross using a better ste6 tester strain. The mex1 allele is designated ste6-2-ts.

The mex2 mutation is not allelic with ste2 or ste6: Two mex2 strains were crossed with α ste2 strain 947 to form diploids K33 and K34, and an α mex2 strain was crossed with RSA21 (a ste6) to form diploid K6ZZ. As shown in Tables 1A.6 and 1A.7 these crosses yielded mating-proficient a segregants, indicating that mex2 is not allelic with ste2 or ste6. 29% of the MATa spores from crosses K33 and K34 were mating

proficient, as were 33% of the MATa spores from cross K6ZZ.

The mex2 mutation is allelic with ste14: Table 1A.8 shows clearly that no mating-proficient MATa segregants were obtained in cross K35. From this I conclude that the mex2 mutation is an allele of STE14, designated the ste14-2 allele.

The temperature-sensitivity of mex1 cosegregates with the sterile phenotype: Table 1A.9 shows that all mating-deficient segregants of cross K22 (α mex1 by a MEX1) are deficient only at high temperature (34°), and that all MATa segregants mate proficiently as a when grown at low temperature (25°). From this I conclude that the conditionality of the mex1 mating defect is an intrinsic property of the mex1 allele and is not due to an unlinked temperature-sensitive modifier of mex1. Quantitative assays of mating efficiency have not been performed on mex1 strains, but they appear to produce wildtype a-factor halos at room temperature (25°). Their a-factor phenotype at 34° is like that of the ste6::lacZ strain described in Chapter 2: a vestigial or no halo is visible.

The third cross to the HR125-5d genetic background: Crosses K37 and K36 were done to introduce the mex1 (ste6-2-ts) and the mex2 (ste14-2) alleles, respectively, into the HR125-5d strain background. The K36 (ste14-2) cross segregated 4 leu^-: 0 Leu^+ as expected, but the K37 cross which should have been heterozygous for leu2 did not give 2:2 segregation. I attribute this to uncertain markers (leu and his particularly) from the mex1 mutant strain, and suggest that the $ste6\text{-}2^{ts}$ allele be picked out of the chromosome using a gapped STE6 plasmid (ORR-WEAVER and SZOSTAK 1983) and transferred via gene replacement into the HR125-5d strain if any further use is to be made of it.

DISCUSSION

The mex1 mutation has been shown by genetic analysis to be a temperature-sensitive allele of ste6 that I have designated ste6-2^{ts}. The phenotype of a ste6-2-ts cells at the nonpermissive temperature (34^{o}) is similar to the phenotype of the ste6::lacZ mutation described in Chapter 2, although quantitative mating assays are required to determine how close to wildtype activity the allele has at the permissive temperature. I showed that the temperature-sensitivity of mating and a-factor production in ste6-2-ts mutants is associated with the mating defect, and suggest that the ste6-2^{ts} gene product is heat-labile either for synthesis or function. If the latter, then this allele may be useful for studies of STE6 function in vivo and in vitro.

The mex2 mutation has been shown to be allelic with ste14, and is designated the ste14-2 allele. Its phenotype is typical of the other ste14 mutants isolated by BLAIR (1979) and myself (Chapter 4), namely sterility in a cells and a severe deficiency in a-factor production.

Limited analysis of the phenotype of α mex3 cells suggests that they are not defective in α-factor synthesis, and therefore that mex3 is either allelic with STE3 or represents a new α-specific STE gene.

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Table 1A.1. Yeast strains

NAME	GENOTYPE	SOURCE
6B55	MATα ade5 his5 ura4 met4 met13 HO$^-$	L. Blair
6B78	MATa (same as 6B55)	L. Blair
70	MATα thr3-10	F. Sherman
227	MATa lys1-1 cryR	J. Hicks
947	MATα ste2-1 ade2-1 lys1-1 hom3 iso1 can1 his2	L. Hartwell
HR125-5d	MATa ura3-52 leu2-3 leu2-112 trp1-am his3 his4	R. Jensen
HR125-11b	MATα leu2-3 leu2-112 ura3-52 trp1-am his3 his4	R. Jensen
HR129-5d	MATα ste14-1 leu2-3 leu2-112 ura4 (his3 and/or his5) met	R. Jensen
K18-6d	MATα mex2 ho ade1 trp1 ura3-52 (arg4?) LEU HIS	
K19-2c	[HO mex3] or [ho MATa mex3] his4 leu2 trp1 (his3? arg4?)	
K19-2d	[ho MATa mex3] or [HO mex3] ade1 leu2 trp1 ura3-52 his4 (his3? arg4?)	
K20-2d	MATα ho mex1 ade1 trp1 HIS LEU	
K22-2a	MATa mex1 ade1 leu2 ura3-52 trp1 (his4 or his3)	
K22-4c	MATα mex1 ade1 ura3-52 trp1-am HIS LEU	
K22-4d	MATα mex1 leu2 trp1 (his3 and/or his4)	
K22-6c	MATa mex1 ade1 leu2 trp1 (his3 and/or his4)	
K27-1c	MATα mex2 ade1 arg4 leu2 trp1 ura3-52 (his3 and/or his4)	
K27-4b	MATa mex2 ade1 trp1 ura3-52 his4 (his3?) LEU	
K27-6b	MATa mex2 ade1 trp1 ura3-52 leu2 his4 (his3?)	
RC757	MATα sst2-1 met1 his6 can1 cyh2	R. Chan
RSA21	MATa ste6-21 ade6 lys2 arg4	J. Rine
T-1851-2D	HO HMRa HMLα ade1 lys2 his4 leu2 trp1 arg4 (the parent strain for the mex mutants)	I. Takano
2-9	mex1 mutant of T-1851-2D	
6-26	mex2 mutant of T-1851-2D	
5-17	mex3 mutant of T-1851-2D	
XMB4-12b	MATa sst1-1 ilv3 arg9 ura1 killer$^+$	L. Blair

Table 1A.2. Crosses

CROSS	PARENTS
K18	mex2 X HR125-5d
K19	mex3 X HR125-5d
K20	mex1 X HR125-5d
K21	RSA21 X K22-4d
K22	K20-2d X HR125-5d
K27	K18-6d X HR125-5d
K29	K22-2a X 947
K30	K22-2a X HR129-5d
K31	K22-6c X 947
K32	K22-6c X HR129-5d
K33	K27-4b X 947
K34	K27-6b X 947
K35	K27-6b X HR129-5d
K36	K27-1c X HR125-5d
K37	K22-4c X HR125-5d
K6ZZ	K27-1c X RSA21

Table 1A.3. *mex1* is not allelic with *ste2*:
a mex1 X α *ste2* crosses

PHENOTYPE at 35°	NUMBER OF SPORES*		
	CROSS K29	CROSS K31	TOTAL
fertile *a*	3	4	7
sterile *a*	22	21	43
fertile α	29	27	56
sterile α	0	0	0

*From six 4-spore and ten 3-spore tetrads for K29, and eight 4-spore, six 3-spore, and one 2-spore tetrads for K31.

Table 1A.4 *mex1* is not allelic with *ste14*:
a mex1 X α *ste14* crosses

	NUMBER OF SPORES*		
PHENOTYPE at 35°	CROSS K30	CROSS K32	TOTAL
fertile *a*	11	13	24
sterile *a*	31	24	55
fertile α	40	35	75
sterile α	0	0	0

*From nineteen 4-spore tetrads for K30, and fifteen 4-spore and four 3-spore tetrads for K32.

Table 1A.5 mex1 is allelic with ste6:
α mex1 X a ste6 crosses

	NUMBER OF SPORES*			
PHENOTYPE**	CROSS K21A	CROSS K21B	CROSS K21C	TOTAL
fertile a	2***	0	0	2
sterile a	19	27	12	58
fertile α	23	22	8	53
sterile α	0	0	0	0

*Includes two 4-spore, fifteen 3-spore, and twenty-eight 2-spore tetrads.

**Assayed at nonpermissive temperature 35°

***Mated reasonably by patch test (>=1% mating) with strain 70 but were still defective in a-factor production.

Table 1A.6 *mex2* is not allelic with *ste2*:
a mex2 X α *ste2* crosses

	NUMBER OF SPORES*		
PHENOTYPE	CROSS K33	CROSS K34	TOTAL
fertile *a*	12	10	22
sterile *a*	19	36	54
fertile α	30	46	76
sterile α	0	0	0

*From twenty-three 4-spore tetrads in K34, and thirteen 4-spore and three 3-spore tetrads in K33.

Table 1A.7 *mex2* is not allelic with *ste6*:
α *mex2* X *a* *ste6* cross

	NUMBER OF SPORES*
PHENOTYPE	CROSS K6ZZ
fertile *a*	2
sterile *a*	3
fertile α	5
sterile α	0

*From two 4-spore and one 2-spore tetrad.

Table 1A.8 *mex2* is allelic with *ste14*:
a *mex2* X α *ste14* cross

	NUMBER OF SPORES*
PHENOTYPE	CROSS K35
fertile *a*	0
sterile *a*	31
fertile α	38
sterile α	0

*Nine 4-spore, seven 3-spore, and six 2-spore tetrads

Table 1A.9 Cosegregation of *mex1* sterility and temperature-sensitivity: α *mex1*^{ts} X *a MEX* cross

	NUMBER OF SPORES*
PHENOTYPE	CROSS K22
fertile *a* at 25°; nonmater at 34°	15
fertile *a* at both temperatures	18
nonmater at both temperatures	0

*From six 4-spore, nine 3-spore, and eight 2-spore tetrads.

APPENDIX 2

DNA SEQUENCE ANALYSIS OF THE 5' FLANKING AND EARLY CODING REGION OF STE6

DNA SEQUENCE DETERMINATION

This appendix consists of figures and tables that provide the details of the DNA sequence determinations using the STE6 5' deletions, as described in Chapter 3. Some of the data presented is essential for future retrieval of plasmids or M13 phage DNA from the Herskowitz freezers.

Table 2A.1: The names of plasmids carrying deletions of STE6 5' DNA.

Table 2A.2: Restriction fragments cloned in M13mp8 and M13mp9 for DNA sequence and deletion endpoint determination.

Figure 2A.1: DNA sequencing strategy.

Figure 2A.2: The published DNA sequence of the 5' end of STE6, numbered 1 through 593 to facilitate reading of the sequencing ladders. Please note that this numbering differs from the corresponding figure in Chapter 3.

Figure 2A.3: DNA sequence ladders corresponding to nucleotides 1-210 (upper strand in Figure 2A.2) and deletion d31 endpoint.

Figure 2A.4: DNA sequence ladders corresponding to nucleotides 204-450 (upper strand in Figure 2A.2) and deletion d67 endpoint.

Figure 2A.5: DNA sequence ladders corresponding to nucleotides 420-592 (upper strand in Figure 2A.2).

Figure 2A.6: DNA sequence ladders corresponding to nucleotides 257-1 (lower strand in Figure 2A.2) and deletion d77 endpoint.

Figure 2A.7: DNA sequence ladders corresponding to nucleotides 490-250 (lower strand in Figure 2A.2).

Figure 2A.8: DNA sequence ladders corresponding to nucleotides 592-460 (lower strand in Figure 2A.2).

Figure 2A.9: DNA sequence ladders corresponding to deletion endpoints for d76, d53, d66, and d62.

Figure 2A.10: DNA sequence ladders corresponding to deletion endpoints for d74, d2, d19, and d61.

TABLE 2A.1. Names of plasmids carrying 5' deletions of STE6 flanking DNA

Deletion name	Orientation I	Orientation II	Plasmid Vector
d31	pC6L-d31	pC6L-31a	YCp50-8-dBH
d61	pC6L-d61-1	pC6L-61	"
d53	pC6L-d53-3	pC6L-53-5	"
d62	pC6L-62	pC6L-62-3	"
d77	pC6L-d77-2	pC6L-d77-3	"
d67	pC6L-d67-2	pC6L-d67-3	"
d19	pC6L-d19b	pC6L-d19a	"
d2	pC6L-d2a	pC6L-d2f	"
d31	p6L-d31-1	not made	YEp13
d53	not made	p6L-d53-3	YEp13

Please note that the plasmid names are similar; C indicates CEN plasmid, as in pC6L-d31. p6L-d31-1 is the same insert, in YEp13.

TABLE 2A.2: Restriction fragments cloned in M13mp8 and M13mp9 for DNA sequence and deletion endpoint determination

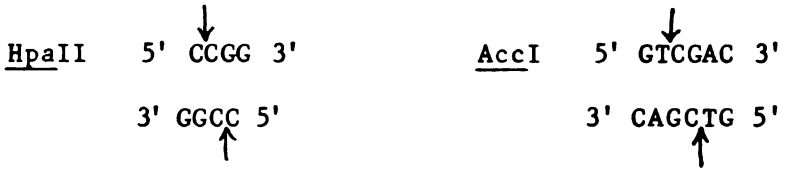
FRAGMENT*	CONSTRUCT NAME	M13 VECTOR	RESTRICTION FRAGMENT**
a	K116-3	mp8	d62-HindIII to RsaI (at 225)
b	K130-22	mp8	d2-HindIII to HpaII
c	K200-6	mp8	d31-HindIII to RsaI (at 225)
d	K201-1	mp9	d31-HindIII to RsaI (at 225)
e	K120-4	mp8	d19-HindIII to RsaI (at 518)
f	K114-1	mp8	d66-HindIII to RsaI (at 225)
g	K150-20	mp8	d74-HindIII to HpaII
h	K161-3	mp9	d77-HindIII to AhaIII
i	K171-2	mp9	d61-HindIII to RsaI (at 225)
j	K113-3	mp8	d76-HindIII to RsaI (at 225)
k	K100-2, -3	mp8	d67-HindIII to HpaII
l	K101-2	mp9	d67-HindIII to HpaII
m	K80-1, -5	mp8	d53-HindIII to HpaII
n	K90-1	mp8	d31-HindIII to HpaII

*Letters refer to fragments shown in Figure 2A.1.

**See next page

Legend to Table 2A.2

**If cloned into mp8, sequenced strand corresponds to upper strand in Appendix Figure 2; if cloned into mp9, sequenced strand corresponds to lower strand. RsaI site locations in nucleotides (Figure 2A.2). Fragments with a HpaII end were cloned into the M13 AccI site via the shared CG sticky ends:



Because the HpaII ends were cloned into AccI sites, the final G nucleotide of the HpaII site is lost. Therefore the sequence ladders only go to nucleotide number 592. I restored the missing G (nucleotide number 593) to the sequence figures (Figure 2A.2).

FIGURE 2A.1. DNA sequencing strategy

Top line depicts the 593-basepair sequenced DNA fragment. Restriction endonuclease sites are abbreviated as follows: A= AhaIII; R= RsaI; HpaII as stated. Where a deletion endpoint is indicated, for example d62, a HindIII site exists in that particular deletion construction, bounded on the left by plasmid vector sequences. Therefore, plasmids carrying deletion d62 retain STE6 DNA to the right of the d62-HindIII site, but not to the left.

The arrows labelled "a" through "n" represent single stranded restriction fragments subcloned into M13 phage vectors mp8 and mp9. If the arrow points to the right, then the fragment was cloned in mp8 and the sequenced strand corresponds to the upper strand of Figure 2A.2. If the arrow points left, then the fragment was cloned in mp9 and the sequenced strand corresponds to the complementary lower strand of Figure 2A.2. Various portions of all the indicated fragments comprise the sequence ladders shown in Figures 2A.3 through 2A.10. The complete sequence of both strands was determined.

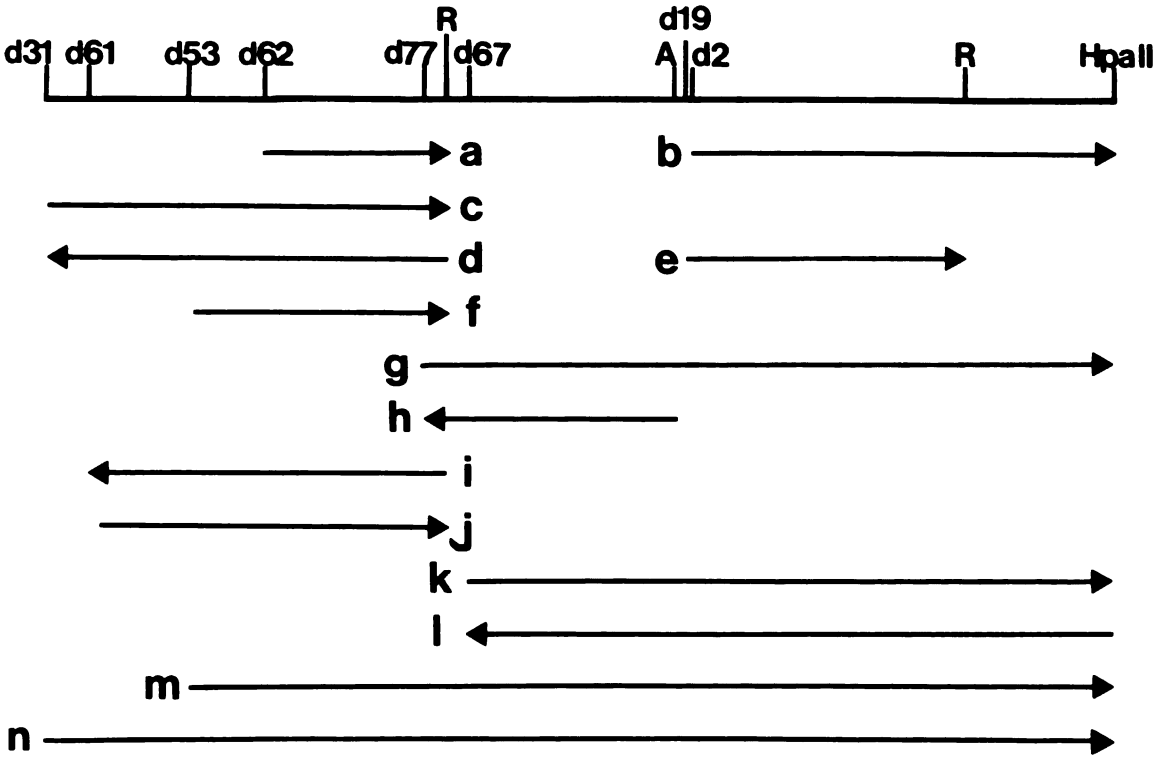


FIGURE 2A-1

FIGURE 2A.2.The DNA sequence of a 593-basepair fragment comprising the 5' flanking and putative N-terminal coding sequence of STE6.

The DNA sequence of the 5' flanking and putative N-terminal coding region of STE6 is shown. d31, d61, etc., refer to BAL31-generated deletion endpoints constructed as described in Chapter 3. A given deletion construction retains all STE6 sequences to the right of the indicated deletion endpoint, and is flanked on the left by a HindIII linker (5' CAAGCTTG 3') and vector sequences.

FIGURE 2A.2. STE6 5' sequence and deletion endpoints.

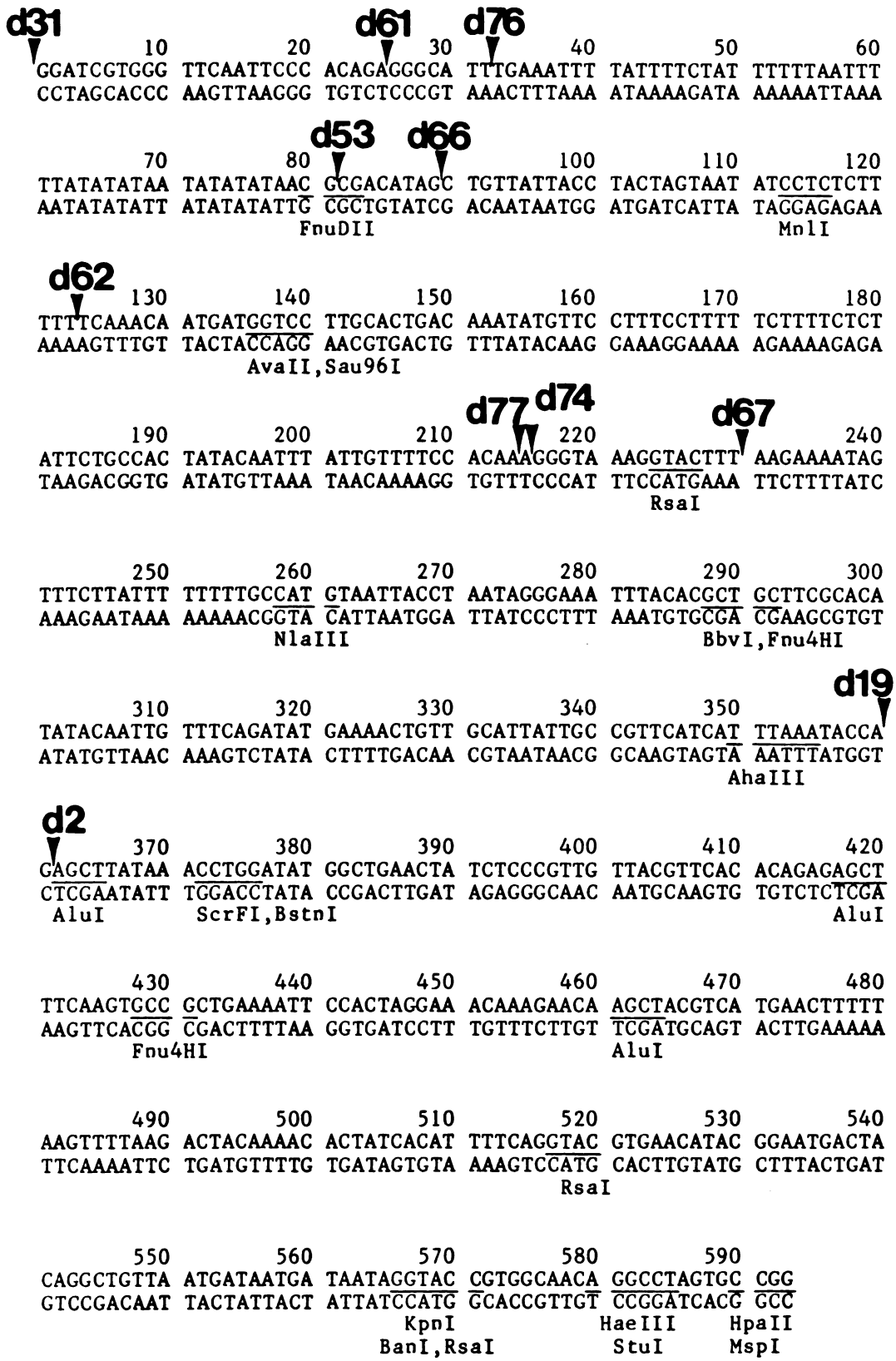


FIGURE 2A-2

FIGURE 2A.3. DNA sequence ladders corresponding to nucleotides 1-210 (upper strand in Figure 2A.2), and deletion d31 endpoint. Named nucleotides (A, G, C, T) correspond to HindIII linker (CAAGCTTG) or M13 polylinker sequences in all of the following DNA sequence figures.

Left: M13 clone K200-6, film F19, primed with 17-mer (see Chapter 3, Methods); deletion d31 defines the most distal end of the sequenced DNA (distal with respect to the STE6 coding sequence).

Middle: M13 clone K90-1, film F11, primed with 17-mer.

Right: M13 clone K80-5, film F9, primed with 17-mer.

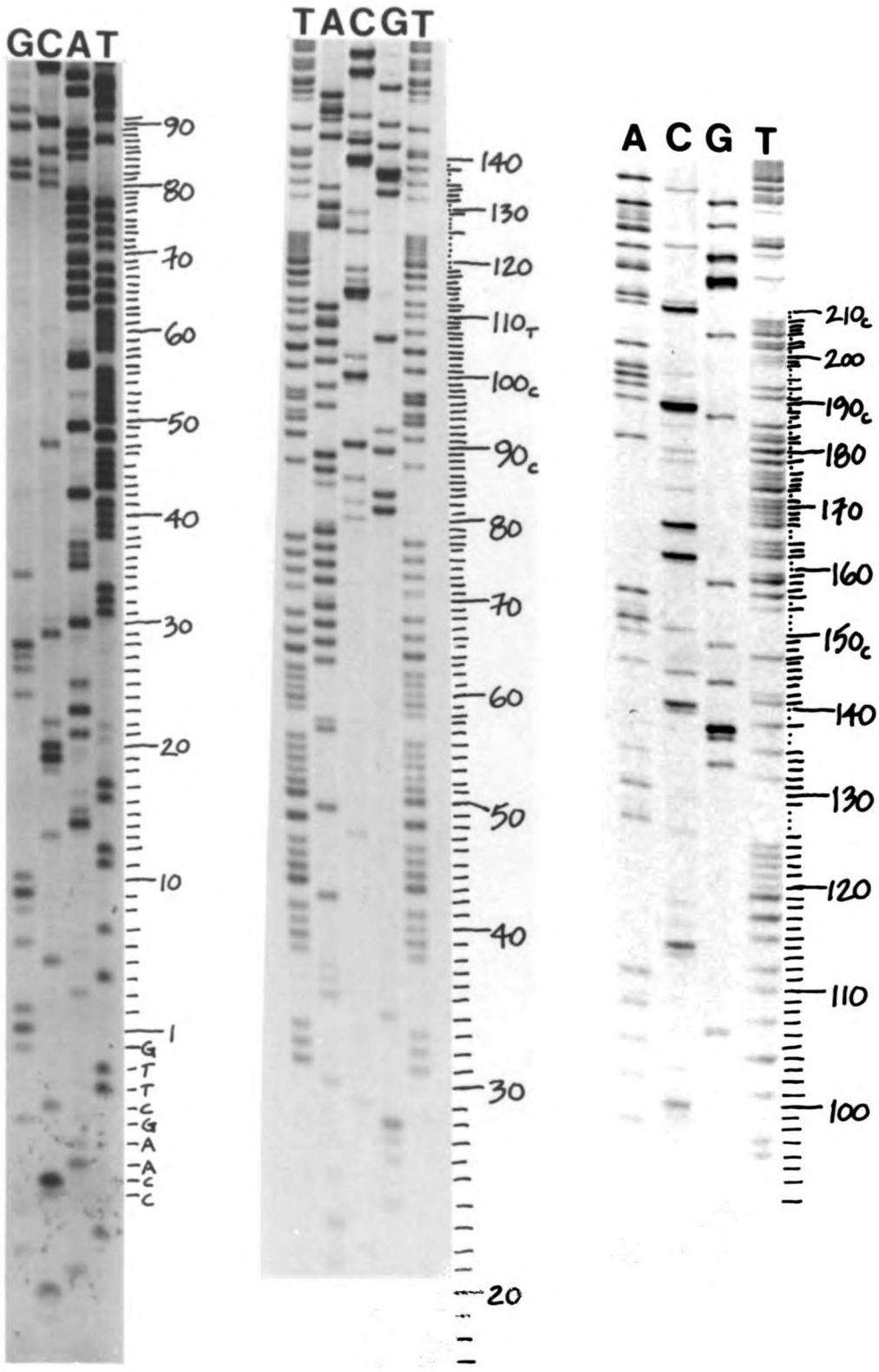

GCAT
90
80
70
60
50
40
30
20
10
1
G
T
T
C
G
A
A
C
C
TACGT
140
130
120
110T
100C
90C
80
70
60
50
40
30
20
A C G T
210C
200
190C
180
170
160
150C
140
130
120
110
100

FIGURE 2A-3

FIGURE 2A.4. DNA sequence ladders corresponding to nucleotides 204-450 (upper strand in Figure 2A.2), and deletion d67 endpoint. GTTCG is part of the HindIII linker.

Left: M13 clone K80-5, film F10, primed with 17-mer.

Middle: M13 clone K100-2, film F1, primed with 17-mer; deletion d67 endpoint.

Right: M13 clone K120-4, film F27, primed with 17-mer.

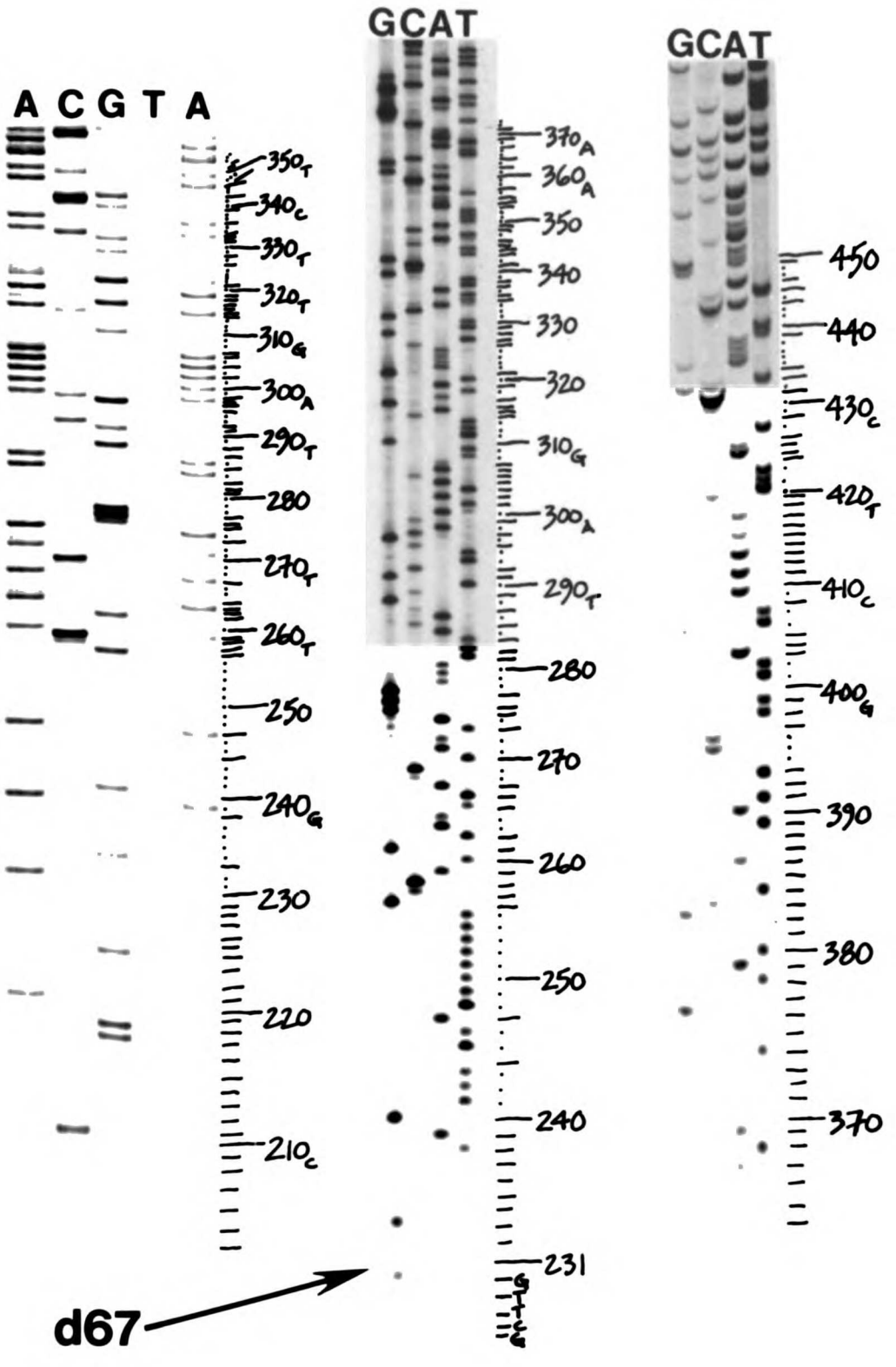


FIGURE 2A-4

FIGURE 2A.5. DNA sequence ladders corresponding to nucleotides 420-592 (upper strand in Figure 2A.2).

Left: M13 clone K130-22, film F31, primed with 17-mer.

Middle: M13 clone K130-22, film F30, primed with 17-mer. The vector sequence beyond the yeast DNA was not identifiable, so a different M13 clone is also shown (next).

Right: M13 clone K100-2, film F32, primed with 17-mer; STE6 sequence ends and M13 polylinker sequences begin.

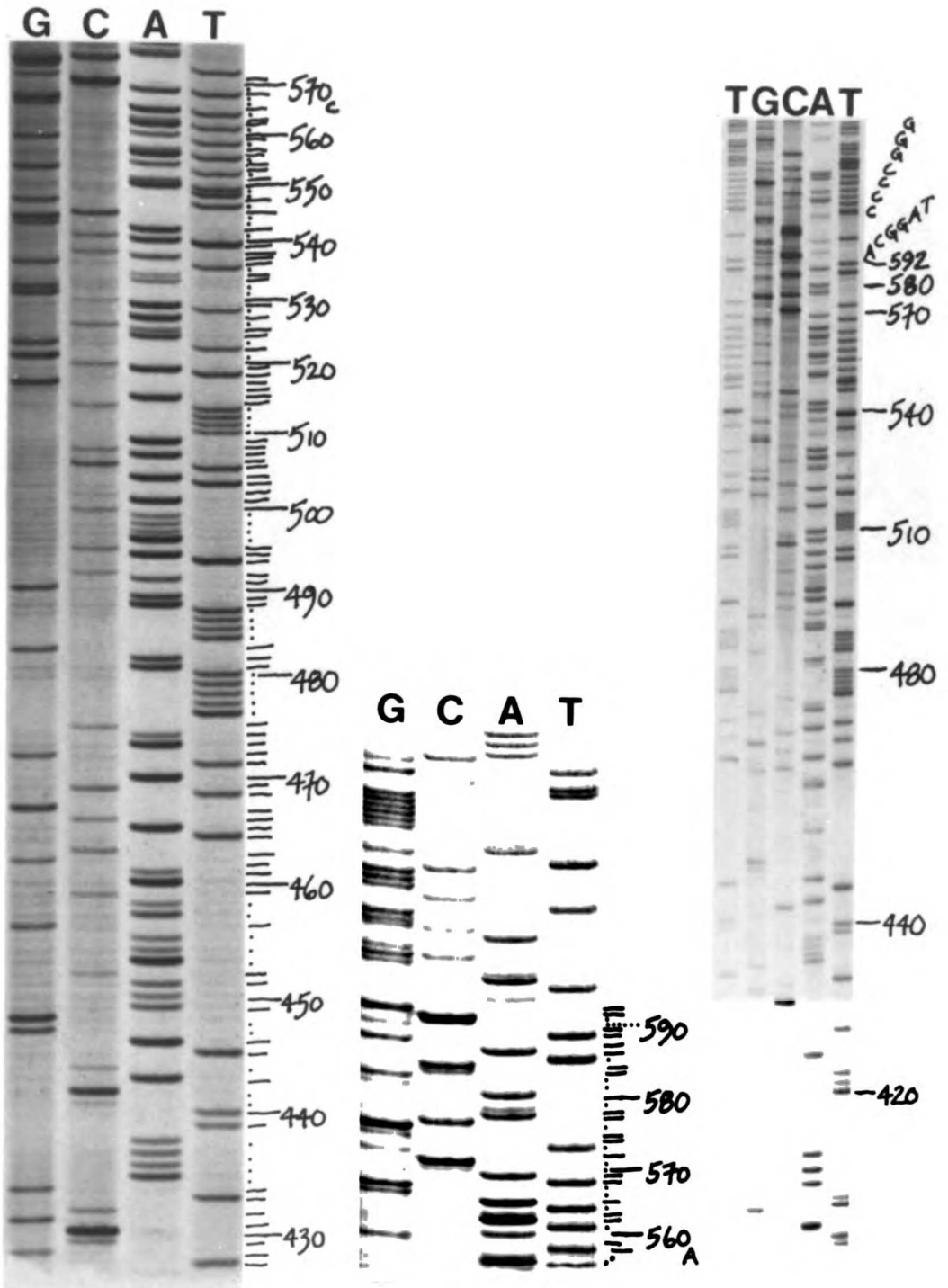

G C A T
570
560
550
540
530
520
510
500
490
480
470
460
450
440
430
G C A T
590
580
570
560
A
TGCAT
592
580
570
540
510
480
440
420

FIGURE 2A-5

FIGURE 2A.6. DNA sequence ladders corresponding to nucleotides 257-1 (lower strand in Figure 2A.2), and deletion d77 endpoint. CAAGCTTG is the HindIII linker adjoining M13 DNA.

Left: M13 clone K201-1, film F18, primed with 17-mer.

Middle: M13 clone K201-1, film F17, primed with 17-mer.

Right: M13 clone K161-3, film F8, primed with 17-mer; deletion d77 endpoint.

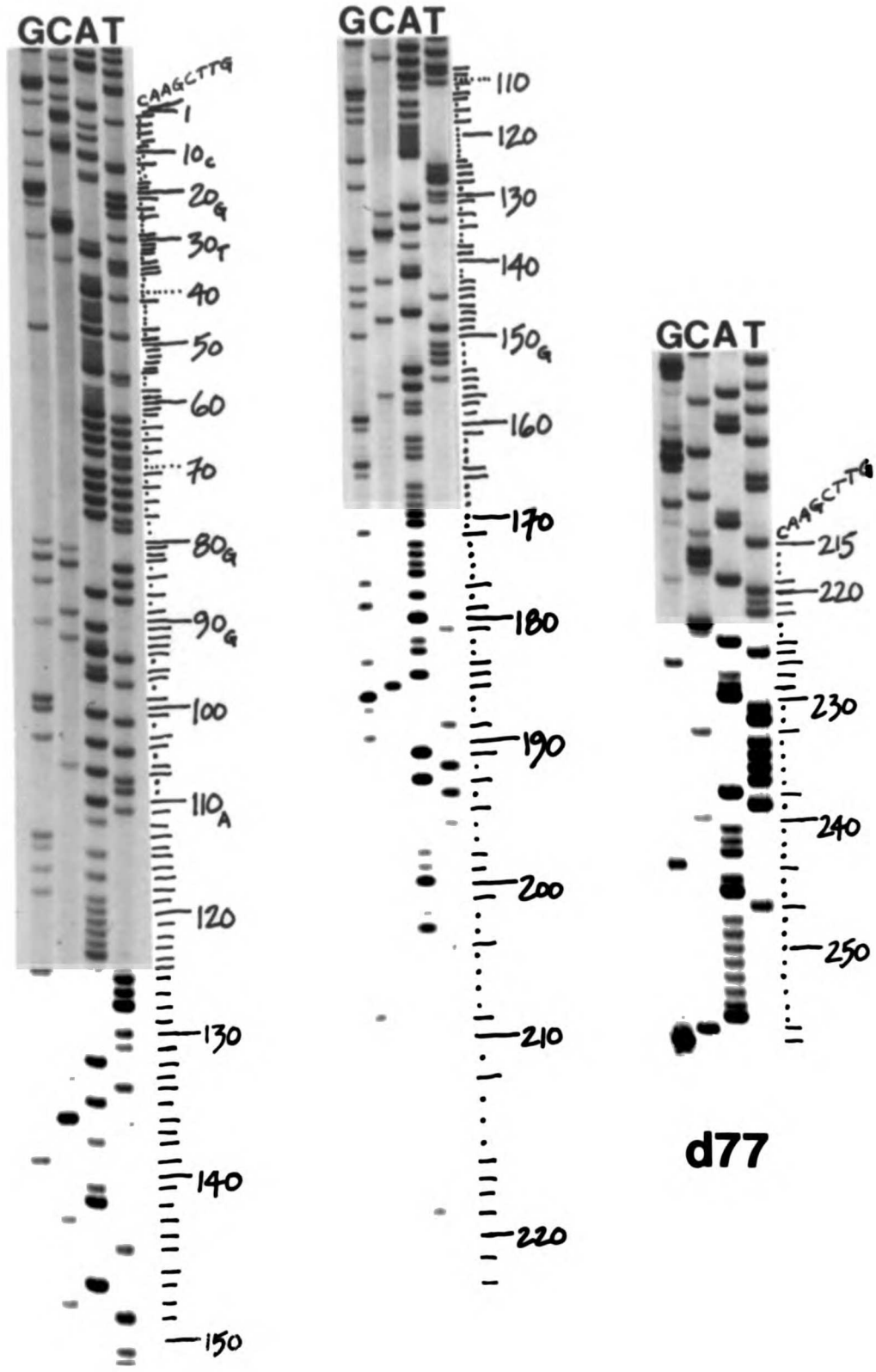

GCAT
CAAGCTTG
1
10C
20G
30T
40
50
60
70
80G
90G
100
110A
120
130
140
150
GCAT
110
120
130
140
150G
160
170
180
190
200
210
220
GCAT
CAAGCTTG
215
220
230
240
250
d77

FIGURE 2A-6

FIGURE 2A.7. DNA sequence ladders corresponding to nucleotides 490-250 (lower strand in Figure 2A.2).

Left: M13 clone K161-3, film F7, primed with 17-mer. This ladder includes a small region where the order of three bands is unclear. The other strand (Figure 2A.4 left and middle) allows unambiguous determination of their order.

Middle: M13 clone K101-2, film F21, primed with 17-mer.

Right: M13 clone K101-2, film F22, primed with 17-mer.

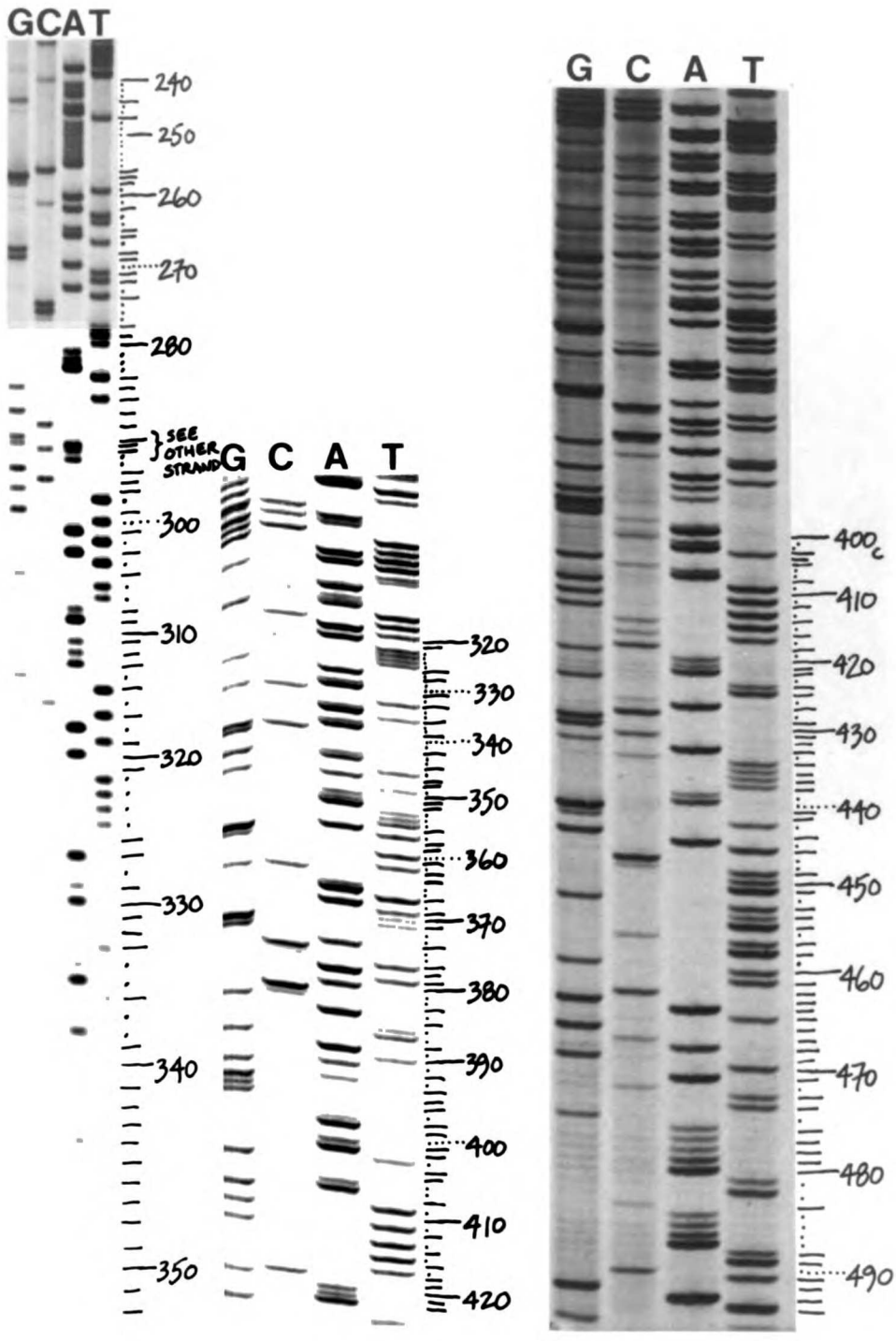

GCAT
240
250
260
270
280
SEE OTHER STRAND
300
310
320
330
340
350
G C A T
320
330
340
350
360
370
380
390
400
410
420
G C A T
400
410
420
430
440
450
460
470
480
490

FIGURE 2A-7

FIGURE 2A.8. DNA sequence ladders corresponding to nucleotides 592-460 (lower strand in Figure 2A.2).

Left (Lower): M13 clone K101-2, film F23, primed with 17-mer.

Right (Upper): M13 clone K101-2, film F2, primed with 17-mer; shows the junction with M13 polylinker sequences better.

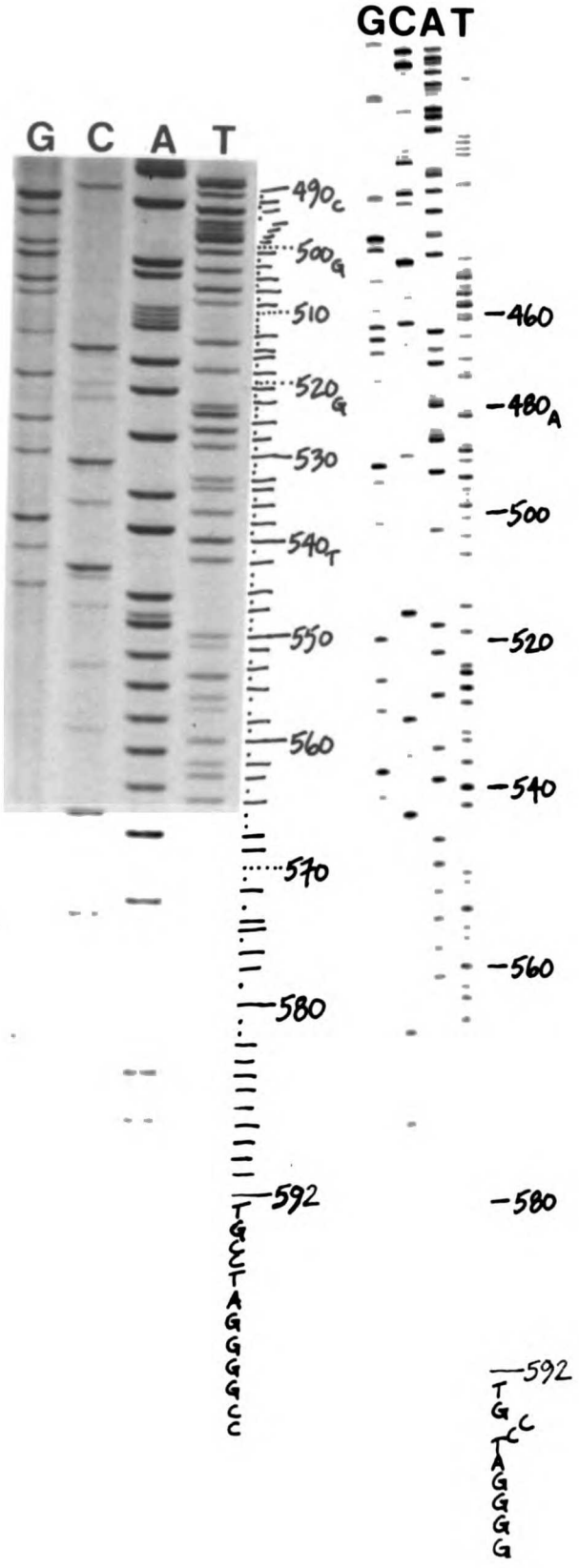

G C A T
490
500
510
520
530
540
550
560
570
580
592
GCAT
460
480
500
520
540
560
580
592

FIGURE 2A-8

FIGURE 2A.9. DNA sequence ladders corresponding to deletion endpoints for d76, d53, d66, and d62. CAAGCTTG is the HindIII linker.

Left: M13 clone K113-3, film F25, primed with 15-mer; deletion d76 endpoint. The 15-mer primer sits further from the site of insertion of the STE6 DNA into the M13 polylinker, and thus provides a better signal for STE6 sequences very close to M13 DNA. It was bad for primary sequence determination because it makes dirty ladders.

Left center: M13 clone K80-1, film F26, primed with 15-mer; deletion d53 endpoint.

Right center: M13 clone K114-1, film F24, primed with 15-mer; deletion d66 endpoint.

Right: M13 clone K116-3, film F13, primed with 15-mer; deletion d62 endpoint.

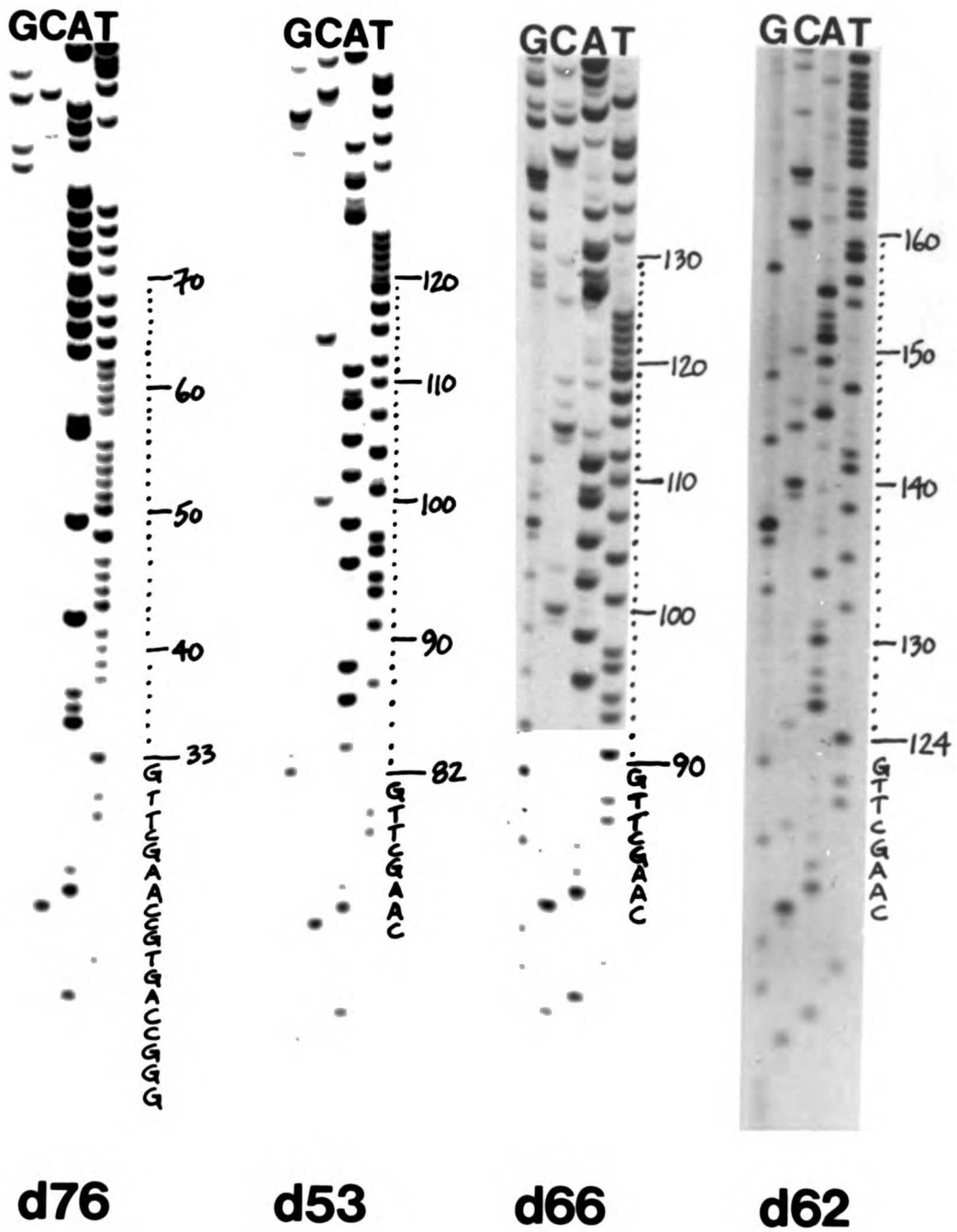

GCAT
70
60
50
40
33
GTTGGAACGGTGACGGGG
d76
GCAT
120
110
100
90
82
GTTGGAAC
d53
GCAT
130
120
110
100
90
GTTGGAAC
d66
GCAT
160
150
140
130
124
GTTGGAAC
d62

FIGURE 2A-9

FIGURE 2A.10. DNA sequence ladders corresponding to deletion endpoints for d74, d2, d19, and d61. CAAGCTTG is the HindIII linker.

Left: M13 clone K150-20, film F6, primed with 15-mer; deletion k74 endpoint.

Left center: M13 clone K130-24, film F5, primed with 15-mer; deletion d2 endpoint. The G nucleotide at the end of the HindIII linker is clearly missing from the d2 junction. The next four nucleotides of the HindIII linker, AGCTT, are exactly the same as the STE6 sequences they replace, resulting in an effective deletion only one base shorter than the k19 deletion.

Right center: M13 clone K120-4, film F14, primed with 15-mer; deletion d19 endpoint.

Right: M13 clone K171-2, film F15, primed with 17-mer; deletion k61 endpoint.

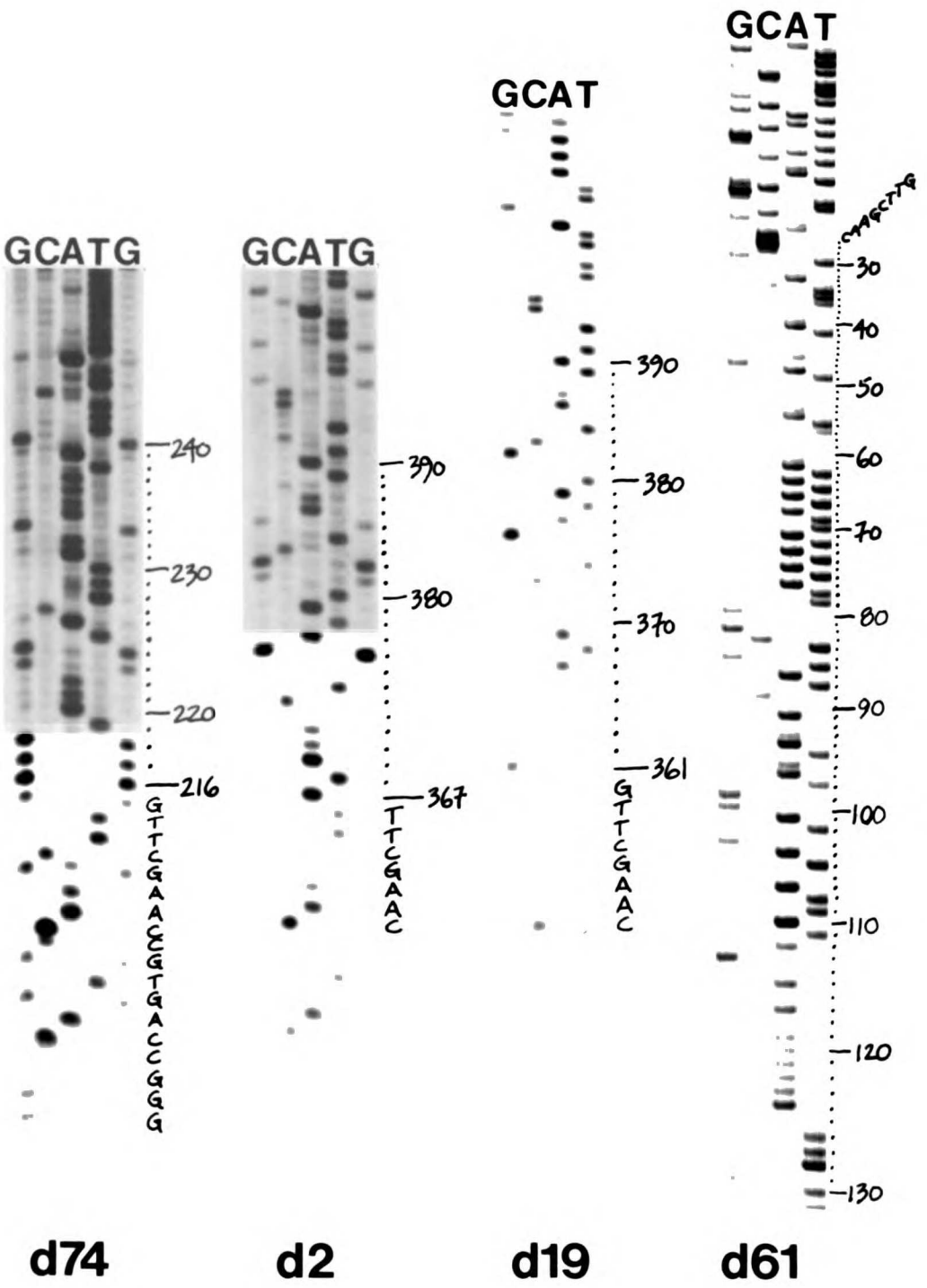

GCATG
240
230
220
216
GTTCGAACGGTGACCGGG
d74
GCATG
390
380
367
TTCGAAC
d2
GCAT
390
380
370
361
GTTCGAAC
d19
GCAT
CAAGCTTG
30
40
50
60
70
80
90
100
110
120
130
d61

FIGURE 2A-10

APPENDIX 3

CLONING OF THE *STE14* GENE AND CREATION OF A POTENTIAL NULL MUTATION

The STE14 gene is required for mating proficiency in a cells but not in α cells. To determine whether STE14 is subject to transcription regulation by the mating type locus, as are STE6 (described in Chapter 2) and the other a-specific genes STE2, MFa1, and BAR1, we isolated the STE14 gene by complementation of the mating defect of an a ste14 mutant strain to use as a probe for STE14 RNA. In addition to gene regulation analysis, the cloned STE14 gene will be used to study STE14 function. The a-factor defect of ste14 mutants is comparable in severity to the defects observed in the ste6::lacZ insertion mutation and the ste16 mutant identified as described in Chapter 4, when assayed by the plate "halo" assay (see Figure 3-3 and Table 3-4 in Chapter 3, and Figure 4-2 in Chapter 4). This appendix describes the isolation and initial characterization of the STE14 gene and is organized as follows:

Section 3 was performed by Candy Waddell while a rotation student in the laboratory.

SECTION 1: The isolation of a DNA fragment that complements the mating defect of a MATa ste14 strain.

Strains, plasmids, media: Yeast strains and crosses are listed in Table 3A.1. Plasmids YCp50-8 and YEp13 are described in Chapters 2 and 3. Plasmids constructed during this work are summarized in Table 3A.2. All media, procedures, strains, and mating type tests are exactly as described in Chapter 2.

Cloning: Strain HR129-2d was transformed (BEGGS 1978) with the Nasmyth clone bank, which consists of random Sau3A-digested yeast DNA fragments in the LEU2 plasmid YEp13. 15,000 independent transformant colonies were obtained by selection for growth on media lacking leucine. Transformants were resuspended in SD (minimal medium plus glucose) and titered prior to screening. However, further screening was not necessary, because a mating-proficient transformant was observed on a titer plate of approximately 2000 colonies that had been tested for mating. The plasmid carried by the fertile colony was isolated by transformation into E. coli and called "p14" (YEp13-STE14).

TABLE 3A.1. Yeast strain list

STRAIN	GENOTYPE	SOURCE
1369	MATα (isogenic with HR125-5d)	R. Jensen
HR129-2d	MATa ste14-1 (3B54 allele of L.C. Blair, Ph.D. thesis, University of Oregon, Eugene, 1979) leu2-3 leu2-112 ade5 can1 cyhR ho	R. Jensen
HR125-5d	MATa ura3-52 leu2-3 leu2-112 trp1-am his3 his4	R. Jensen
K36-6b	MATa ste14-2 (mex2 allele; see Appendix 1) arg4 his4 his3? leu2 trp1-am ura3-52	K. Wilson
K48-4b	MATα ste14-2 ura3 leu2-3 leu2-112 ade2-1-oc trp1-am his3 his4 $SUP4\text{-}3^{ts}$	K. Wilson
K55-1d	MATa ura3-52 [stable p14-9::URA3 integrant] leu2-3 leu2-112 trp1-am his3 his4	K. Wilson

DIPLOIDS	PARENTS
K55	HR125-5d X 1369 [integrated p14-9]
K58	K48-4b X K55-1d

TABLE 3A.2. STE14 plasmids

NAME	VECTOR	YEAST DNA INSERT	SOURCE
p14	YEp13	original insert ca. 4.6 kb	K. Wilson
p14-9	YIp5	4.3-kb BamHI fragment in BamHI site of YIp5	K. Wilson
pC1	YCp50-8	4.3-kb BamHI fragment in BamHI site of YCp50-8	C. Waddell
pC1-4	YCp50-8	TRP1 inserts, with or without deletion of STE14 DNA, into pC1; see Figure 3A.2	C. Waddell
pC1-17	YCp50-8		C. Waddell
pC1-23	YCp50-8		C. Waddell
pC1-27	YCp50-8		C. Waddell

SECTION 2: The cloned DNA fragment integrates at and is therefore homologous with the chromosomal STE14 locus.

The majority of the original insert was subcloned as a 4.3-kb BamHI fragment into the URA3 plasmid YIp5, to form plasmid "p14-9". Plasmid p14-9 was used to transform ura3$^-$ strain 1369, which is the α version of the isogenic HR125-5d series. Ura$^+$ transformants were selected by growth on medium lacking uracil, and a colony that exhibited a stable Ura$^+$ phenotype was identified by its ability to grow on selective medium after prolonged growth on YEPD. The YIp5 vector does not replicate in yeast (unless an ARS is provided by the insert DNA) and is thus not stable unless integrated into the chromosome via its yeast insert homology. If the 4.3-kb insert indeed contains the STE14 gene, then the plasmid (with URA3) will integrate at the chromosomal STE14 locus. The ura3-52 mutation prevents integration at the ura3 locus.

The α strain carrying the integrated p14-9 plasmid was mated with isogenic strain HR125-5d to form diploid K55, sporulated, and dissected. The point of cross K55 was to assure 2:2 segregation of the integrated URA3 marker prior to analyzing its allelism with ste14. In seven complete tetrads, the URA3 marker segregated 2:2 as expected for a properly integrated plasmid. Fertile segregant K55-1d (MATa [integrated URA3] ura3-52) was then crossed with strain K48-4b (α ste14 ura3) to form diploid K58. If the plasmid had integrated at the STE14 locus, then all fertile a spores should be Ura$^+$ and all sterile a spores should be Ura$^-$. Indeed, the URA3 marker was allelic with the ste14 in 18 complete tetrads, as shown in Table 3A.3.

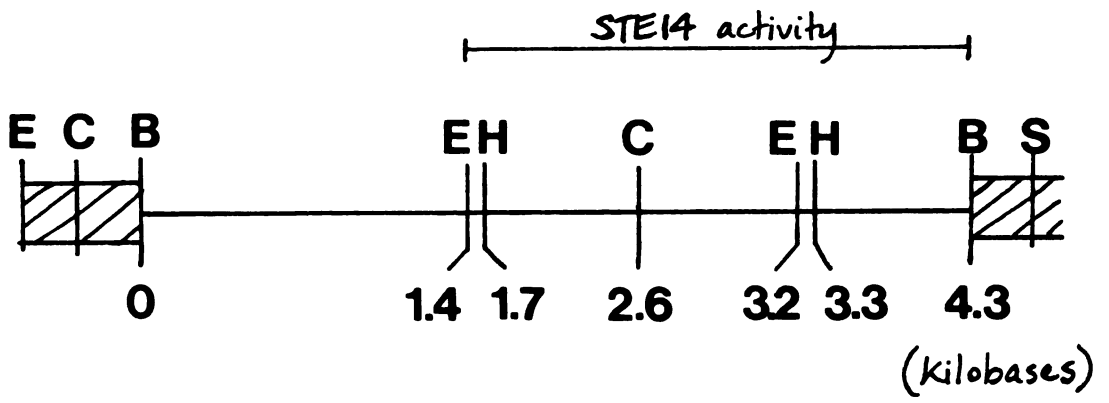
TABLE 3A.3. Plasmid p14-9 integrates at the STE14 locus

Cross K58: a ura3-52 [URA3 at STE14] X α ura3 ste14

Phenotype	Number of Spores
a Ste^- Ura^-	19
a Ste^- Ura^+	0
a Ste^+ Ura^-	0
a Ste^+ Ura^+	17
α Ura^-	17
α Ura^+	19

SECTION 3: Restriction mapping and localization of STE14.

Figure 3A.1 shows the restriction map of the 4.3-kb BamHI fragment shown by C. Waddell to have STE14 complementing activity in the low copy vector YCp50-8. All sizes are approximate (estimated from agarose gels). The relative positions of the HindIII and EcoRI sites have not been confirmed. Vector sequences are indicated by hatched bars. Restriction sites are abbreviated as follows: C= ClaI; H= HindIII; E= EcoRI; B= BamHI; S= SalI. There is at least one XmaIII site present in the insert which has not been mapped. The following enzymes do not cut within the insert: NruI, PstI, SalI, SmaI, SphI, XbaI, XhoI.



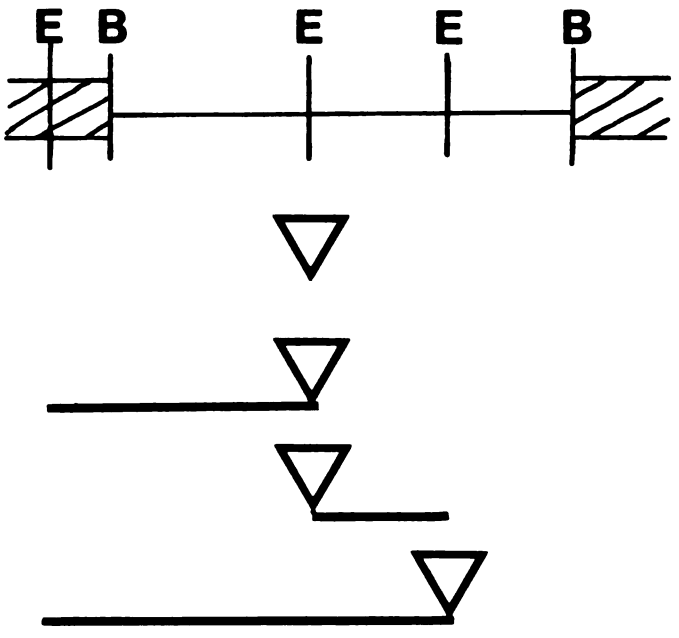
We wanted to localize the STE14 gene to a precise segment of the cloned DNA. The following strategy was used to obtain insertions (of the TRP1 gene) and deletions within the 4.3-kb fragment. Plasmid pC1 (URA3) was subjected to partial EcoRI digestion. Appropriate partial digests were ligated with a 1.45-kb TRP1 EcoRI fragment obtained by EcoRI digestion of plasmid YRp7-MAT. The ligated DNA mixtures were transformed into E. coli strain MH5 (Trp^- Ura^-), selecting for growth on ampicillin. Amp^R colonies were replica plated to M63 minimal medium to screen for cells with plasmids carrying both TRP1 and URA3. Trp^+ Ura^+ colonies were purified and their plasmids isolated by miniprep and characterized by restriction digestion. The expected classes of TRP1 insertions and combined insertions/deletions were unambiguously distinguishable by separate digestions with ClaI and BamHI. Four constructions resulted (see Figure 3A.2), called pC1-4, pC1-17, pC1-23, and pC1-27.

The effect of the TRP1 insertions/deletions on the STE14 complementing activity of each plasmid was determined by transformation into strain K36-6b (a ste14-2 trp1-am) and testing the mating phenotype of the yeast transformants. This information is summarized in Figure 3A.2.

The orientation of the TRP1 insert within each plasmid has not been determined. An EcoRI digest of each to confirm the predicted loss of discrete EcoRI fragments has not been done. However, the complementation results make a prediction as to the location of STE14 (see section 5) which can be tested directly.

FIGURE 3A.2. Complementation assays with altered plasmids

Vector sequences are indicated by hatched bars, deleted DNA is represented by solid bars, and TRP1 insertions at EcoRI sites are indicated by triangles. E= EcoRI, B= BamHI, C= ClaI.



NAME	COMPLEMENTS ste14⁻ ?
pC1-4	yes
pC1-23	yes
pC1-17	no
pC1-27	no
pC1	yes

STE14+

SECTION 5: Summary.

The DNA fragment isolated by complementation contains the STE14 gene because 1) it directs the integration of plasmid YIp5 to a locus that is allelic with ste14 (see Table 3A.3), and 2) it complements the mating defect of a ste14 strain when carried on the low copy vector YCp50-8 (see Figure 3A.2).

We have determined a rough restriction map of the STE14-containing fragment, shown in Figure 3A.1. By insertion and insertion/deletion analysis of the cloned fragment, we have delimited the complementing activity to the 2.9-kilobasepair region encompassing the ClaI site in Figure 3A.1. Very little work will be required to determine exactly where the STE14 gene resides, for the purposes of making null mutations or insertional fusions (for instance with lacZ) of STE14 in the chromosome. If STE14 is located within the central 1.8-kb EcoRI fragment, then plasmid construction pC1-17 is a null mutation that can easily be replaced into the wildtype STE14 allele in the chromosome.

The question remains whether STE14 is regulated by the mating type locus, in particular, whether its transcription is repressed by α2. One attempt at analyzing total (not poly(A)-enriched) RNA showed no discrete band regulated by mating type (data not shown). The experiment is being repeated with poly(A)-enriched RNA and a different internal control gene.

Strain K55-1d, or derivatives of it, may be useful for mapping STE14 in yeast since the STE14-linked URA3 marker may be followed in all cell types.

APPENDIX 4

CLONING OF A PUTATIVE STE16 GENE

This appendix describes the presumptive cloning of the STE16 gene by Pablo Garcia during his rotation in the Herskowtiz lab, by complementation of the mating defect of a MATa ste16-1 strain. The identification of the ste16 gene was described in Chapter 4. All procedures, plasmid vectors, bacterial strains and mating type tests are described in Chapters 2 and 3, except that the Carlson clone bank in YEp24 (URA3, 2§ high copy) was used rather than the Nasmyth bank. In addition, α strain XT1175-S245C was used as a mating type tester. Two distinct DNA inserts were isolated; which of these two contains the true STE16 gene has not yet been determined.

Table 4A-1. Strains

NAME	GENOTYPE	SOURCE
K91-3b	MATa cryR ste16-1 ura3-52 leu2-3 leu2-112 lys2-oc tyr1-oc trp1-am his4	K. Wilson
K91-3b	with plasmid pYPG1	P. Garcia
K91-3b	with plasmid pYPG2	P. Garcia
K91-3b	with plasmid pYPG3	P. Garcia
HR125-5d	MATa ura3-52 leu2-3 leu2-112 trp1-am his3 his4	R. Jensen
1369	MATα (isogenic with HR125-5d)	R. Jensen
HR129-2d	MATa ste14-1 ade5 leu2-3 leu2-112	R. Jensen
K39-3b	MATa ste6-21 leu2-3 leu2-112 his4 ade6 lys2 can1	K. Wilson
XT1175-S245C	MATα ade6 his6 leu1 met1 trp5-1 gal2 can1 rme	

Isolation of plasmids that complement the mating defect of a ste16 mutant: Strain K91-3b was transformed with the Carlson YEp24 clone bank (M. Carlson, pers. comm.) and transformants were selected by growth on media lacking uracil. A screen of 500 colonies for mating ability by replica plating showed, surprisingly, that about 1% of the colonies were mating-proficient and made wildtype a-factor halos. Eleven mating-proficient colonies were restreaked and tested anew for mating ability both before and after loss of the plasmid (colonies become Ura^- after growth on rich medium). Ten colonies retested as mating proficient had the auxotrophic markers of the parent strain, and were dependent upon the plasmid for both mating proficiency and a-factor production. Plasmid DNA was isolated by transformation of E. coli (either MH1 or MC1066) and analyzed by restriction digestion with EcoRI. Three classes of plasmids were found, called pYPG1 (recovered six times), pYPG2 (recovered twice), and pYPG3 (found once). Restriction mapping indicated that pYPG1 (3.7 kb insert) was a subset of pYPG2 (6.9 kb insert), and that pYPG3 was unrelated to both of them. Restriction maps of the three plasmids are shown in Figure 4A-1.

The plasmids have not yet been retransformed into yeast to confirm their STE16 complementing activity. Are there really two different plasmids that can complement the ste16 defect? If so, one may contain STE16 and the other could contain a gene with related but different function that could give us a clue about STE16. The true STE16 gene probably resides within pYPG1, simply because two independent plasmids with this insert were recovered many times. The restriction maps of the potential STE16 plasmids are different from those of STE6 (Chapter 2), STE14 (Appendix 3), MFa1 and MFa2 (BRAKE et al. 1985; Chapter 4).

Table 4A-2. Testing for complementation with heterologous plasmids.

		MATING WITH*		PRODUCTION OF	
STRAIN	PLASMID	227 (a)	70 (α)	a-factor	α-factor
ste14 mutant					
HR129-2d	none	-	-	-	-
HR129-2d	YEpSTE6	-	-	-	-
HR129-2d	YEpSTE14	-	+	+	-
ste6 mutant					
K39-3b	none	-	-	-	-
K39-3b	YEpSTE6	-	+	+	-
K39-3b	YEpSTE14	-	-p	-	-
ste16 mutant					
K91-3b	none	-	-	-	-
K91-3b	YEpSTE6	-	-p	-	-
K91-3b	YEpSTE14	-	-p	-	-

*Symbols used: +, mating (prototrophic patch on minimal medium); -, no mating (no growth on minimal medium); -p, no growth of patch on minimal medium, but a few colonies do grow, indicating rare matings are occurring. Results are based on mating tests on five independent transformants of each type.

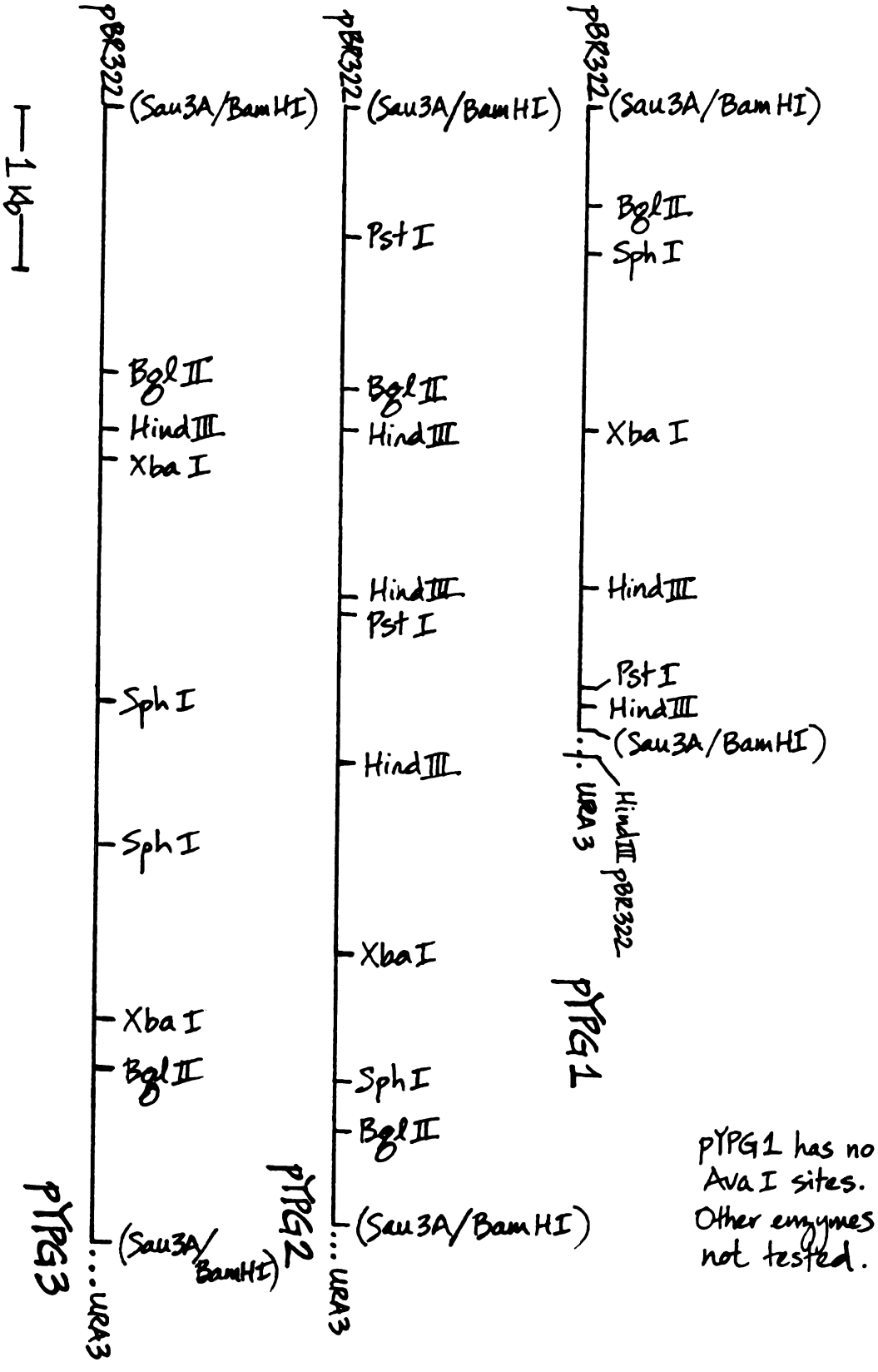

1 kb
pBR322
(Sau3A/BamHI)
BglII
HindIII
XbaI
SphI
SphI
XbaI
BglII
(Sau3A/BamHI)
URA3
pYPG3
pBR322
(Sau3A/BamHI)
PstI
BglII
HindIII
HindIII
PstI
HindIII
XbaI
SphI
BglII
(Sau3A/BamHI)
URA3
pYPG2
pBR322
(Sau3A/BamHI)
BglII
SphI
XbaI
HindIII
PstI
HindIII
(Sau3A/BamHI)
URA3
HindIII pBR322
pYPG1
pYPG1 has no AvaI sites.
Other enzymes not tested.

FIGURE 4A-1

The mating defects of ste6, ste14, and ste16 strains are complemented only by plasmids carrying their wildtype cloned gene and not some other genes present in high copy: To determine if the *STE6* and/or the *STE14* gene products, when overexpressed, could suppress the *ste16* defect, the *ste16* strain K91-3b was transformed with *LEU2* 2§ YEp13 plasmids carrying *STE6* ("YEp13-STE6"; Chapter 2) and *STE14* ("p14"; Appendix 3). I believe that the *STE6* gene product is actually expressed at high levels from the YEp13 vector because β-galactosidase activity from a YEp13 *ste6::lacZ* fusion is expressed at 50-100 times the level of a *ste6::lacZ* gene in the chromosome. I do not know that the *STE14* gene product is actually expressed at high levels when it is carried by YEp13.

To determine if either the *ste6* or the *ste14* mating defects could be complemented by high copy suppression from the other cloned wildtype gene, strain K39-3b (*a ste6-21*) was transformed with the *STE14* plasmid p14, and strain HR129-2d (*a ste14-1*) was transformed with the *STE6* plasmid YEp-STE6. The results are shown in Table 4A-2 and indicate that each strain is fully complemented only by its wildtype cloned gene and not by cloned genes for the other *a*-factor biosynthetic functions. I conclude that *STE6*, *STE14*, and *STE16* gene products perform nonoverlapping functions in the biosynthesis of *a*-factor pheromone.