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Role of Intron Splicing in the Function of the *MAT***a***1* Gene of *Saccharomyces cerevisiae*

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The *MAT*a*1* gene of *Saccharomyces cerevisiae* is unique in yeast cells in that it contains two short intervening sequences (IVS1 and IVS2) 54 and 51 nucleotides long. The 3′ intron is inefficiently spliced and results in the accumulation of transcript with only the first intron removed, leading to the speculation that the gene may produce different protein products by alternative splicing patterns. We have used in vitro mutagenic techniques to construct intronless *MAT*a*1 genes and have introduced point substitutions in the 5′-TACTAAC-3′ internal conserved sequence of each intron to identify the protein product that is required for repression of haploid-specific genes. Analysis of these constructs for the ability to repress expression of an *HO*::*lacZ* fusion and for the ability to allow diploid cells to undergo sporulation during conditions of starvation revealed that the gene is functional with two, one, or no introns and that the only functional protein is the one produced when both introns are spliced from the mRNA.

Three distinct cell types are found in the yeast *Saccharomyces cerevisiae*, a diploid cell type, **a**/α, and haploid cell types, **a** and α, which are distinguished by cell-type-specific phenotypes such as the ability to mate, pheromone production and response, meiosis, and sporulation, all of which are determined by the expression of unlinked genes. In **a** cells, **a**-specific genes are expressed (e.g., *STE2*, component of the receptor to α-factor; *BAR1*, protease for the degradation of α-factor; and *MFA1* and *MFA2*, structural genes for **a**-factor precursor); in α cells, α-specific genes are expressed (e.g., *STE3*, component of receptor to **a**-factor; and *MFα1* and *MFα2*, structural genes for α-factor precursor). Diploid cells express a set of genes that allow meiosis and sporulation to occur, but the **a**- and α-specific genes are not expressed, and hence these cells are unable to mate. The *MAT* locus, a single genetic locus on chromosome III, determines which set of these genes is expressed. Haploid **a** and α cells carry *MAT***a** and *MAT*α information, respectively, and diploid cells, which result from the mating of **a** and α cells, are heterozygous for *MAT* and carry both loci (for reviews, see references 13 to 15).

The *MAT***a** and *MAT*α loci contain unique Ya and Yα regions (1) that encode two proteins in *MAT*α (α1 and α2) and one protein in *MAT***a** (**a**1) (26). These proteins determine cell type by regulating expression of the cell-type-specific unlinked genes. Strathern et al. (37) advanced the α1-α2 hypothesis to explain cell-specific regulation by the *MAT* locus. The model proposed that in **a** cells, the **a**-specific genes are expressed without any action by *MAT***a**. In α cells, the product of *MATα1* is a positive regulator of α-specific genes, and the *MATα2* gene product is a negative regulator of **a**-specific genes. A set of haploid-specific genes, including those necessary for mating, is expressed in both **a** and α cells. In **a**/α diploids, the products of *MAT***a***1* and *MATα2* genes act together to turn off the haploid-specific genes, including the *MATα1* gene, the absence of whose product switches off α-specific genes. Also, the gene product of *MATα2* represses **a**-specific genes. The net result is that the **a**/α diploid cell is unable to respond to the mating pheromones but has the ability to enter meiosis and activate sporulation-specific genes (25). This repression is brought about by the interaction of **a**1 and α2, as heteromeric proteins, with sequences found upstream of haploid-specific genes (e.g., *HO* and *RME1*). Specifically, **a**1 is required to alter the binding activity of α2 from that of sequences found upstream of **a**-specific genes to those of sequences found upstream of haploid-specific genes, thus conferring a dual-repressor function on α2 (10).

Sequence analysis of the *MAT***a***1* gene (1) together with S1 mapping of the transcripts (23) revealed that the gene, unlike any other characterized nuclear gene in yeasts, contains two introns. Both introns contain 5′-GTATGT. . . . .AG-3′ splice sequences at the positions predicted by the S1 mapping data and also the essential 5′-TACTAAC-3′ internal conserved sequence (16, 19, 31). However, it was observed that during mRNA processing, the 3′ intron was not excised as efficiently as the 5′ intron, suggesting that the splicing intermediate with only the first intron removed may also be translated to produce a functional protein (23). Both the putative polypeptides coded by *MAT***a***1*, with and without the second intron removed (**a**1 and **a**1′, respectively), share homology with procaryotic DNA-binding proteins (20, 35). It is therefore possible that the *MAT***a***1* gene encodes two proteins of related function, which leads to the speculation that there are two **a**1/α2 species (**a**1′/α2 and **a**1/α2). To identify which of the polypeptides encoded by *MAT***a***1* is utilized for haploid-specific repression, all possible combinations of the intron deletions were constructed, and a set of point changes was introduced in the TACTAAC sequence of the two introns. In vivo repression studies and primer extension analysis with use of these constructs suggest that both introns must be spliced efficiently and correctly to produce a functional *MAT***a***1* protein. However, none of the introns is required for the production of a functional *MAT***a***1* product.

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TABLE 1. Oligonucleotides used[a]

Name	Sequence	Characteristic
SN1	5′-aatctttataattttataatggaa-3′	Deletion of IVS1
SN2	5′-agtaagagtttggttcataaataa-3′	Deletion of IVS2
SN3	5′-ttctaggtactgagat-3′	Sequencing primer for IVS1
SN10	5′-tatcctatC\ActaG\C\Acaattt-3′	Point mutations in TACTAAC of IVS2
SN13	5′-caagcacgggcatttt-3′	Sequencing primer for IVS2
SN24	5′-tagatctcatacgttt-3′	Primer extension
SN25	5′-caatttcctaacaatac-3′	Point mutation in IVS1

[a] The position of the oligonucleotide in the *MAT***a***1* gene is underlined in Fig. 1. Oligonucleotide SN10 was used to introduce point substitutions at two positions, indicated by capital letters, by synthesis of a degenerate sequence (see Materials and Methods).

MATERIALS AND METHODS

Strains. *Escherichia coli* K-12 RR1 (F *hsd520* [r_B^- m_B^-] *ara-14 proA2 lacY1 galK2 rpsL20* [Str^r] *xyl-5 supE44* λ^- *leuB6 thi recA*$^+$) was the host strain for propagating yeast shuttle vectors and for isolation of DNA for subcloning and yeast transformations. *E. coli* JM101 [*supE thi* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}ZΔM15)] (38) and *E. coli* RZ1032 {HfrKL16 PO/45 [*lysA*(*61-62*)] *dut ung thi-1 relA1*} (17, 18) were the host strains for isolation of single-stranded non-uracil-containing and uracil-containing DNAs, respectively.

S. cerevisiae SNY1 (*MAT*α *HO*::*lacZ trp1 met*$^-$ *ura3*) was a segregant of a cross between RP123 (*MAT*α *his2 ade1 trp1 met14 ura3*) (obtained from S. Roeder) and K1107 (*HMR***a** *MAT***a** *HML***a** *ura3 HO*::*lacZ46 ade2-1 can1-100 met*$^-$ *his3 leu2-3 leu2-112 trp1*) (obtained from L. Breeden and K. Nasmyth). The diploid strain SNY3 (*MAT*α/*mat*::*CAN HIS3/his3 leu2/leu2 ade1/ADE1 ura3-52/ura3-52 TRP1/trp1 gal/GAL*) was isolated after mating NA-36 (*MAT*α *ura3-52 leu2-3,112 ade1 gal*) (obtained from A. Hopper) with MH52-3C (*mat*::*CAN trp1 his3 leu2 ura3-52 rme*) (33). S704 (*MAT***a** *his4-912 spt2-150 ura3-52 lys1-1 leu2-3*) (obtained from S. Roeder) was used as the tester stain in assessments of mating efficiency.

Media and biochemicals. All *E. coli* strains were grown in yeast extract-tryptone (YT) broth, and yeast strains were grown in selective dropout medium or yeast extract-peptone-dextrose (YPD) broth. *E. coli* DNA polymerase (Klenow fragment), T4 DNA ligase, and T4 polynucleotide kinase were obtained from Promega Biotec. [α-^{32}P]- and [γ-^{32}P]dATP were purchased from Amersham International. Unlabeled deoxynucleotides and dideoxynucleotides were obtained from Pharmacia, Inc.

Plasmid constructs. Oligonucleotide mutagenesis was performed on M13mp11 bearing a 4.2-kilobase (kb) *Hin*dIII fragment of the *MAT***a** locus. This fragment was then subcloned into the yeast centromere-containing shuttle vector YCp50 (constructed by M. Johnston and R. W. Davis). YCpSN10-26, YCpSN10-32, YCpSN10-40, and YCpSN25-13 contain 3.5-kb *Eco*RI-*Hin*dIII fragments of the *MAT***a** locus subcloned into a modified YCp50 (YCpΔ) that has no *Bgl*II, *Sal*I, *Sph*I, and *Bam*HI sites.

Transformations. Competent *E. coli* cells were prepared by the calcium chloride procedure (21). Yeast cells were transformed by the spheroplasting procedure described by Sherman et al. (36).

Sporulation and mating analysis. The diploid strain SNY3 was transformed with the YCp50 recombinant plasmids containing the mutant *MAT***a** loci, and the transformants were patched onto potassium acetate plates or grown on liquid sporulation medium and incubated at 30°C for 3 to 5 days (37). Spore formation was assessed under a phase-contrast microscope. The procedure described by Hartwell (12) was used to mate SNY1 strains containing variant *MAT***a***1* constructs with a *MAT***a** tester strain, S704.

Synthesis and purification of oligonucleotides. All oligodeoxynucleotides (sequences are listed in Table 1) were synthesized on an Applied Biosystems model 380A DNA synthesizer. After synthesis, all oligodeoxynucleotides were deprotected and purified as described by Atkinson and Smith (2).

Preparation of single-stranded DNA and single-stranded DNA containing uracil. Single-stranded M13 DNA was prepared essentially as described by Sanger et al. (34). Preparation of DNA containing uracil (18) has been described by Ner et al. (28). This uracil-containing DNA was used without further purification for mutagenesis.

Oligonucleotide-directed mutagenesis. For deletion of introns from the *MAT***a***1* gene and for introduction of point substitutions into the 5′-TACTAAC-3′ sequences of the two introns of the *MAT***a***1* gene, the mutagenesis protocol described by Ner et al. (28) was used. A single oligonucleotide (SN10; Table 1) was synthesized to generate the three changes indicated for the intervening sequence IVS2. During the synthesis cycle, a mixture of C and A nucleotide monomers (1:1) at position 9 and a mixture of G, C, and A monomers (1:1:1) at position 13 of the oligonucleotide was incorporated to generate a mixed population of oligonucleotides (22). All mutations were screened by dideoxy sequencing as described by Sanger et al. (34) and Ner et al. (27).

Rescue of plasmids from yeast strains. The yeast strain harboring the *MAT***a** construct on the shuttle vector was grown overnight in 5 ml of selective medium. Total yeast DNA was isolated by a modification of the method described by Sherman et al. (36) and suspended in 100 μl of TE (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA). One-tenth of this mixture was used to transform competent *E. coli* RR1 cells, plasmid DNA was isolated from ampicillin-resistant colonies as described by Maniatis et al. (21), and the region spanning the deletions or substitutions was sequenced (4).

Preparation of yeast mRNA. Yeast mRNA was prepared essentially as described by A. Spence (Ph.D. thesis, University of British Columbia, Vancouver, British Columbia, Canada, 1986). A single colony of yeast cells harboring the desired shuttle vector was grown overnight in selective medium (5 ml); 1 ml of this culture was used to inoculate 100 ml of selective medium, and the preparation was incubated at 30°C to an A_{530} of 0.6 to 0.8. Cycloheximide was added to the culture (final concentration, 0.1 mg/ml), which was then left at room temperature for 5 min. The culture was chilled on ice for 5 min and transferred to chilled centrifuge tubes. The cells were harvested at 3,000 × *g*, suspended in 1 ml of ice-cold water containing cycloheximide (0.1 mg/ml), and transferred to Eppendorf tubes. The cells were again centri-

fuged, and the supernatant fraction was removed by aspiration. The pellets were immediately frozen in dry ice-ethanol. Acid-washed glass beads that had been treated with trimethylchlorosilane were added to the frozen cells (2 g of beads per g [wet weight] of cells), followed by 2 ml of ice-cold extraction buffer (0.15 M NaCl, 0.1 M Tris hydrochloride [pH 7.5]) and 50 μl of vanadyl ribonucleoside complexes per ml. Cells were broken by vigorous vortexing for six 15-s intervals, each followed by 45 s of cooling on ice. After centrifugation at 15,000 × *g* for 10 min at 4°C, the supernatant was transferred to a clean tube, and sodium dodecyl sulfate (SDS) and proteinase K were added to concentrations of 0.5% and 0.5 mg/ml, respectively. The cell debris was extracted a second time after resuspension in RNA extraction buffer (2 ml) and vanadyl ribonucleoside complexes (50 μl/ml). After centrifugation, the supernatant was combined with the first fraction, and the SDS concentration was adjusted to 0.5%. The combined extracts were incubated at 37°C for 1 h. An equal volume of 4 M LiCl was added, and the mixture chilled overnight on ice to precipitate high-molecular-weight RNA. The precipitate was collected by centrifugation (10,000 × *g*, 20 min), rinsed with cold 2 M LiCl–10 mM EDTA, and then dissolved in water. The RNA was isolated by ethanol precipitation from 0.3 M sodium acetate, rinsed with cold ethanol, dried, and suspended in water.

Primer extension. Primer extension was performed by using a synthetic oligonucleotide (SN24) that had been phosphorylated at the 5′ end with [γ-^{32}P]ATP. The protocol followed was essentially that described by Domdey et al. (6), with minor modifications. After incubation with avian myelocymatosis virus reverse transcriptase, the mixture was phenol extracted, ethanol precipitated, dried, suspended in 10 μl of formamide-dye mix (same as used for dideoxy sequencing) and 50 mM NaOH (2.5 μl), and incubated at 42°C for 15 min. The sample was then boiled and loaded onto a 6% polyacrylamide–7 M urea sequencing gel.

β-Galactosidase assays. The method used for β-galactoside assays has been described by Russell et al. (33). SNY1 bearing the constructs was grown on selective medium to near stationary phase, optical density at 600 nm was recorded, and a 100-μl sample of the culture was treated with chloroform (50 μl) and 0.1% SDS (25 μl) at 30°C for 5 min. The substrate, *o*-nitrophenylgalactoside (ONPG; 200 μl; 4 mg/ml) was added, and the mixture was incubated at 30°C until sufficient color had developed. The reaction was stopped by the addition of 1 M sodium bicarbonate (500 μl), the cell debris was centrifuged to a pellet, and the A_{420} of the supernatant was measured. Units of activity were calculated as described by Miller (24).

RESULTS

Deletion of introns from the *MAT*a*1* gene. Evidence from S1 nuclease studies suggested that the two introns of the *MAT*a*1* gene are differentially spliced (23). The 3′ intron is spliced less efficiently than the 5′ intron, suggesting the existence of two polypeptide products, one terminating at the stop codon within IVS2 (a1′) and the second terminating beyond IVS2 when that intron has been correctly removed (a1). We sought to define which of the protein products were involved in repressing haploid-specific genes. The strategy adopted was (1) to generate constructs containing deletions of the intron and (2) to generate constructs containing point changes at the internal conserved sequences (TACTAAC [19]) of both introns and hence disrupt correct splicing. The ability of the constructs to restore repression of haploid functions was then assessed.

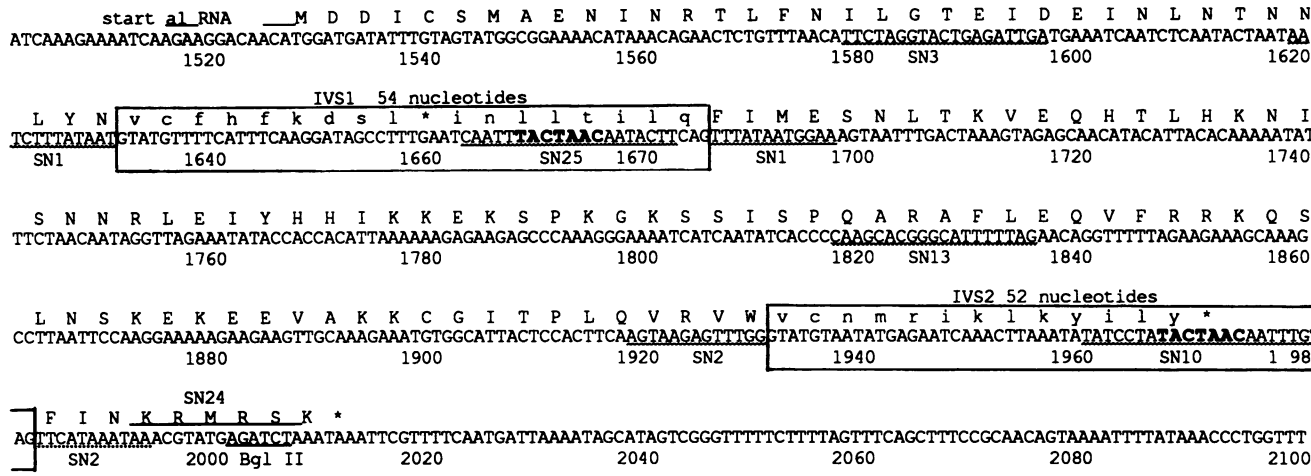
The *MAT*a locus on a 4.2-kb *Hin*dIII fragment (1) was subcloned into the *Hin*dIII site of M13mp11. Single-stranded DNA containing uracil was isolated by passage through *E. coli* RZ1032 (*dut ung*) (18) and used in oligonucleotide-directed deletion of IVS1 and IVS2. Two oligonucleotides (SN1 and SN2, both 24 nucleotides long) were synthesized to direct the deletions. SN1 removed 54 nucleotides and SN2 removed 52, corresponding to the regions between the natural 3′ and 5′ splice sites (Fig. 1; 23). The construct bearing a deletion of IVS1 (M13mpSN1) was then used to prepare single-stranded DNA containing uracil, which served as a template for SN2-directed deletion of IVS2. The desired deletions in the *MAT*a*1* gene were verified by dideoxy sequencing of the coding region. The 4.2-kb *Hin*dIII fragments for the three constructs were transferred to the yeast shuttle vector YCp50 and are designated YCpSN1, YCpSN2, and YCpSN1-SN2 (Fig. 2).

Analysis of *MAT*a*1* deletion constructs. All constructs were assessed for the ability to produce a functional a1 polypeptide that in the presence of α2 should repress the expression of an *HO*::*lacZ* gene fusion, allow diploid cells to sporulate, and prevent mating with a tester a strain when the construct is carried in an α strain. The yeast *MAT*α strain SNY1, isolated as a segregant from a cross between the a strain K1107 and an α strain, RP123, contains an *HO*::*lacZ* fusion inserted at the chromosomal location of the *HO* locus (33). When this strain is transformed with the *MAT*a locus present on a plasmid, the isolates are heterozygous for *MAT*. Functional a1 and α2 proteins are able to repress expression of the fusion. Transformants carrying constructs bearing the mutations of *MAT*a*1* were assayed for β-galactosidase activity (Table 2). The strain harboring the vector alone was unable to repress the fusion. Plasmids YCpSN1 (IVS1 deleted), YCpSN2 (IVS2 deleted), and YCpSN1-SN2 (both introns deleted) produced similar levels of β-galactosidase activity to YCpWt (wild-type *MAT*a*1*), showing that the *MAT*a*1* variants were functional in repression of the *HO* promoter and that the introns were not required for gene function.

To assess the ability of the *MAT*a*1* intron deletions to allow diploid cells to sporulate, a *MAT*Δ/*MAT*α strain (SNY3) was transformed with the mutant plasmids. The *MAT*a locus on the plasmid confers heterozygosity at the *MAT* locus; in the presence of α2, a1 is able to repress *RME1*, a haploid-specific gene that inhibits entry into meiosis (25). Sporulation was observed for all deletion constructs (Table 2), confirming that all three mutants, lacking either or both of the introns, were as functional as the wild-type gene. Mating with a tester a strain was similarly prevented when these constructs were present in an α background (Table 2).

Construction of mutations in the TACTAAC sequences of IVS1 and IVS2. A second method of determining the functionality of polypeptides resulting from differential splicing of the *MAT*a*1* transcript was to disrupt the correct splicing of the intervening sequences. As indicated earlier, there was a significant amount of RNA in yeast cells in which the 3′ intron (IVS2) had not been removed so that two products (Fig. 1, a1 and a1′) arising from the differential splicing of IVS2 (YCpWt and YCpSN1) could result. Because the two products from this differential splicing are present together, the contribution of either of the products alone, as *trans*-acting factors, cannot be assessed. By preventing splicing of IVS2, the product (a1′) terminating at the stop codon within this intron will be the only one present and thus can be evaluated for function. The *MAT*a*1* introns contain a 5′-

A



B

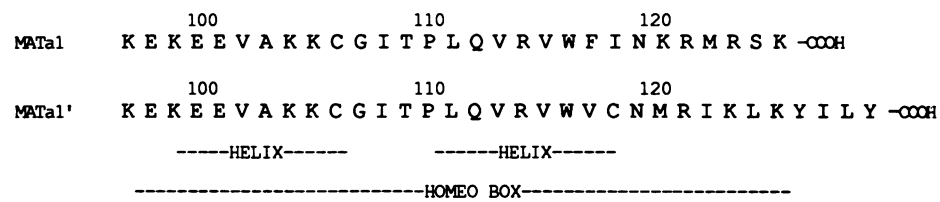


FIG. 1. (A) Sequence of the *MATa1* gene showing the positions of intervening sequences. The two introns are boxed, and the internal conserved sequences are in boldtype face. The predicted protein sequence when both introns are spliced is in capital letters. The predicted translation sequence within the introns is in lowercase letters. Asterisks identify termination codons. The unbroken lines above the nucleotide sequence in the 5′ region indicate the two initiation sites for transcription, separated by 9 nucleotides. The wavy lines below the sequence correspond to regions for which oligonucleotides were synthesized. The line above the nucleotide sequence corresponds to the oligonucleotide complementary to mRNA used in the primer extension analysis of the *MATa1* transcripts. The nucleotide-numbering system is that of Astell et al. (1). (B) Predicted polypeptide sequences for *MATa1* and *MATa1′* arising when IVS2 is spliced and unspliced, respectively. The putative proteins share a common first helix of the helix-turn-helix motif but differ within and beyond the second helix. They both share similarities with the homeobox domain sequence.

TACTAAC-3′ that is required for formation of the spliceosome complex (3, 5, 9, 11). This sequence contains the branchpoint nucleotide for lariat formation (6, 32). Single point mutations in this sequence, particularly at the branchpoint A nucleotide (TACTAAC), disrupt correct excision of the intervening sequences (6–8, 16, 29, 30) through disruption of spliceosome assembly (3, 9, 11). Therefore, mutations were introduced in the IVS2 TACTAAC to prevent splicing of the intron. This procedure would result in the formation of only one protein product that utilizes the coding sequence up to a termination codon within IVS2.

We constructed YCpSN10-26, YCpSN10-32, and YCpSN10-40, which are deleted for IVS1, and contain point mutations in IVS2, and have the sequences 5′-TACTACC-3′, 5′-TCCTAAC-3′ and 5′-TACTAGC-3′, respectively (Fig. 2). The UAA of the UACUAAC is an in-frame termination codon in IVS2. In YCpSN10-26, this codon was changed to a UAC and should allow translation to proceed to the ochre codon present 15 residues beyond the 3′ splice site of IVS2. In construct YCpSN10-40, the termination codon was changed to a UAG, and hence no readthrough beyond this point should occur. The ability of these constructs to repress the *HO*::*lacZ* fusion and to allow sporulation to take place in a *MATΔ*/*MATα* diploid was assessed (Table 2). The levels of β-galactosidase activity observed for YCpSN10-26, YCpSN10-32, and YCpSN10-40 were similar to that observed with the vector alone, suggesting that the **a**1 protein variants arising from these constructs were unable to repress *lacZ* transcription and hence were not functional. When these constructs were present in an α strain and mated with a tester **a** strain, mating was observed at normal levels, suggesting the polypeptide **a**1′ present does not function to block mating.

YCpSN25-13 has IVS2 removed and contains a single change in IVS1 (5′-TCCTAAC-3′). With this mutant, we observed 30 to 50% repression of the *HO*::*lacZ* fusion, sporulation in *MATΔ*/*MATα* diploid strains bearing this construct, and mating with a tester **a** strain when YCpSN25-13 was carried in an α strain (Table 2), suggesting that a sufficient amount of functional **a**1 polypeptide was present to repress *RME1* but not to prevent mating. We do not have a definite explanation of this result. As mentioned earlier, splicing of this intron was more efficient than that of the 3′ intron, IVS2. Therefore, it is possible that the mutant is leaky. In addition, the intron is 54 nucleotides long, and hence an unspliced RNA would be in frame for translation. However, translational readthrough of this intron to produce a variant **a**1 protein with an insert of 18 amino acids would also require readthrough of a nonsense codon centrally positioned in IVS1 (1). As demonstrated below, the mutant

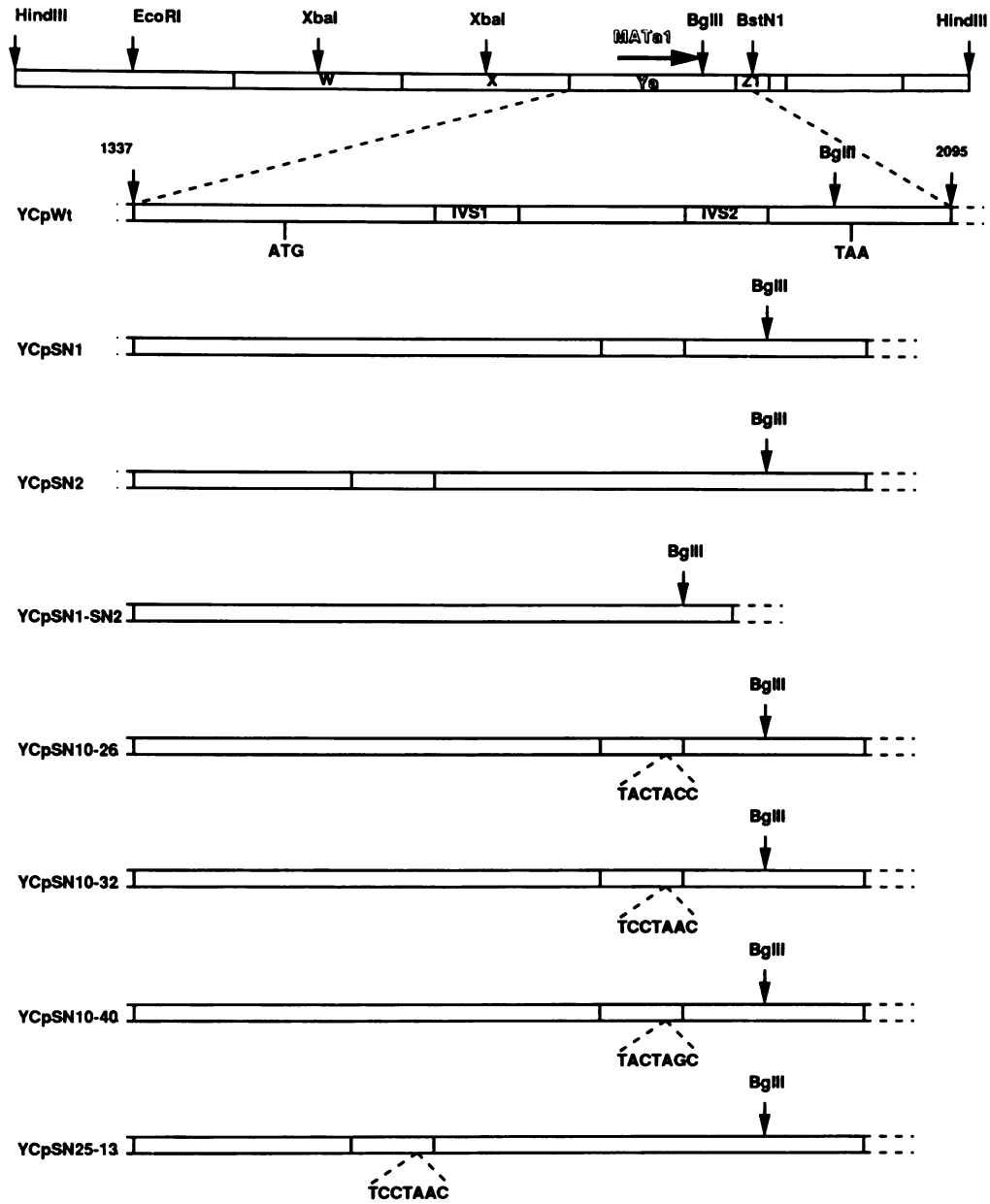


FIG. 2. Summary of plasmids containing altered *MAT*a*1* genes. A 4.2-kb *Hin*dIII fragment of the *MAT*a locus was cloned into the yeast shuttle vector YCp50. The region containing the *MAT*a*1* gene is expanded to show the positions of the intervening sequences and the point substitutions introduced into the TACTAAC sequence of either IVS1 or IVS2. The numbering system is that of Astell et al. (1).

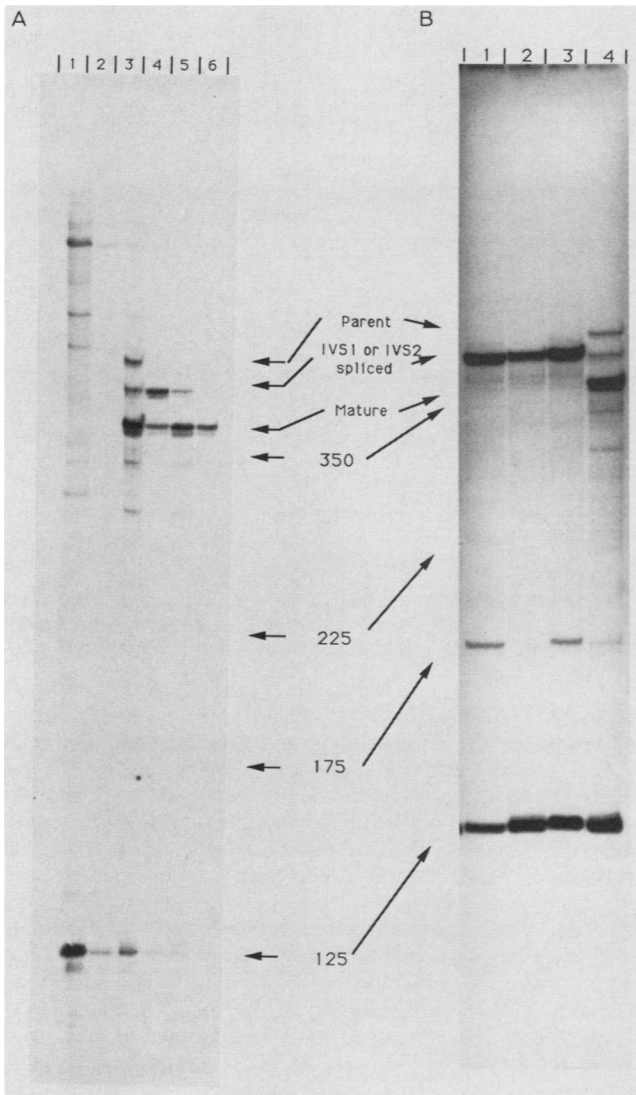
gene does produce a small amount of mature-length mRNA, and this apparent leakiness is the best available explanation of this observation.

Primer extension analysis: the product from the correctly spliced transcript is needed for repression of haploid functions. The results from in vivo function analysis indicate whether the mutant *MAT*a*1* gene product is functional but do not identify the product. In the constructs containing point substitutions in mRNA-splicing signals, there may be leakiness and production of mature wild-type mRNA. To investigate this possibility and to show that splicing was disrupted as desired, the mRNA from the mutants was isolated, and primer extension analysis was carried out by using an oligonucleotide complementary to the 3′ end of the message (SN24; Table 1 and Fig. 1). The results for the deletion mutants are shown in Fig. 3A. Strain SNY1 contains a disruption of the *MAT*a locus and contains no functional *MAT*a*1* product. No transcript corresponding to the *MAT*a*1* message was observed (lane 1). However, this strain showed two major unidentified extension products, one corresponding to 130 nucleotides and the other to more than 900 nucleotides, and several other minor transcripts. These transcripts were present in all cases but at lower levels (lanes 1 to 6). The strain carrying the wild-type *MAT* locus (YCpWt; lane 3) showed three major bands corresponding to parent transcript (493 nucleotides), mature message (387 nucleotides), and the RNA that has one intron unspliced (441 nucleotides). These bands appeared as doublets corresponding to the two sites of initiation of transcription (26). The intensities of the three bands indicated that the mature

TABLE 2. β-Galactosidase activity, sporulation analysis, and mating efficiency of *MATa1* constructs carried in strains SNY1 and SNY3

Construct	Change[a]	Activity[b] (±10%)	Mating efficiency[c]	Sporulation[d] (%)
YCp50		100	0.91	0
YCpWt	None	5	1.9×10^{-5}	62
YCpSN1	I1 deleted	5	4.3×10^{-6}	49
YCpSN2	I2 deleted	5	6.9×10^{-6}	63
YCpSN1-SN2	I1 and I2 deleted	5	4.7×10^{-5}	53
YCpSN25-13	I1 TCCTAAC, I2 deleted	40	0.27	49
YCpSN10-26	I2 TACTACC, I1 deleted	95	0.45	0
YCpSN10-32	I2 TCCTAAC, I1 deleted	95	0.57	0
YCpSN10-40	I2 TACTAGC, I1 deleted	95	0.84	0

[a] Change introduced into the *MATa1* gene. I1, IVS1; I2, IVS2. The underlined nucleotide indicates the substitution introduced into the TACTAAC sequence.
[b] Relative level of β-galactosidase activity of the chromosomal *HO::lacZ* fusion in SNY1. The basal level of activity in SNY1 was 12 U (24).
[c] Values for constructs in SNY1 mated with tester a strain, S704. Values are normalized to that for the efficiency of mating of strains SNY1 and S704, which was 7.4×10^{-1}, and are averages of two experiments.
[d] Observation of tetrad formation. The constructs were transferred to a diploid strain that contained a nonfunctional *MATa1* gene (SNY3). Four transformants for each construct were grown on sporulation medium and analyzed for the presence or absence of tetrads after incubation for 5 days. Values are from the observation of more than 500 cells carrying each construct.



message was the predominant species, whereas the parent transcript and RNA containing one unspliced intron were present in approximately equal amounts. The primer extension profile for YCpSN1 (lane 4), which has IVS1 deleted, showed only two bands, one corresponding to the parent transcript of 441 nucleotides, containing a single intron, and the other to the mature message of 387 nucleotides, with no intron present. The major product corresponded to that containing the intron (IVS2), showing that this intron was inefficiently processed. YCpSN2 (lane 5), which contains IVS1 and lacks IVS2, gave the same type of profile. However, the intensities of the bands were opposite those observed with YCpSN1, demonstrating the more efficient splicing of IVS1. These results on splicing efficiency are in agreement with those of Miller (23). The construct with both intervening sequences removed yielded a single RNA (lane 6) corresponding to the mature message observed with YCpWt (lane 3).

FIG. 3. Primer extension analysis of intron-deletion constructs and point substitutions in the TACTAAC sequences of the *MATa1* gene. Total RNA (approximately 50 μg) was used as template for reverse transcriptase primer extension, using a ^{32}P-labeled oligonucleotide (SN10) as primer. (A) Lanes: 1, RNA template from an α strain, SNY1, containing a nonfunctional *MATa1* gene; 2, RNA template from SNY1 bearing the yeast shuttle vector YCp50; 3, RNA template from SNY1 containing the wild-type *MATa* locus inserted into YCp50 (YCpWt); 4, RNA template from SNY1 bearing the *MATa* locus, with deletion of IVS1 in the *MATa1* gene, inserted into YCp50 (YCpSN1); 5, RNA template from SNY1 bearing the *MATa* locus, with deletion of IVS2 in the *MATa1* gene, inserted into YCp50 (YCpSN2); 6, RNA template from SNY1 containing the *MATa* locus, with deletion of IVS1 and IVS2 in the *MATa1* gene. (B) Lanes: 1, RNA template from SNY1 bearing the *MATa* locus inserted into YCp50, containing a deletion of IVS1 and a point substitution (TACTAAC to TACTACC) in IVS2 of the *MATa1* gene (YCpSN10-26); 2, RNA template from SNY1 bearing the *MATa* locus inserted into YCp50, which contains a deletion of IVS2 and a point substitution (TACTAAC to TCCTAAC) in IVS1 of the *MATa1* gene; 3, RNA isolated from SNY1 containing the *MATa* locus (the *MATa1* gene is deleted for IVS1 and contains a point substitution [TACTAAC to TACTAGC] in IVS2 [YCpSN10-40]); 4, RNA isolated from SNY1 containing the wild-type *MATa* locus (YCpWt) inserted in YCp50. Primer extension products were purified and electrophoresed on polyacrylamide-urea sequencing gels, after which the ^{32}P-labeled products were detected by autoradiography. A sequencing ladder (not shown) was also electrophoresed adjacent to the products to determine the lengths of the transcripts.

In addition to the parent transcript and transcripts with one or both introns removed, some shorter products were observed. These were presumably intermediates in splicing or alternate splicing products, because they were present only when at least one of the introns was present. The splicing of IVS1 (YCpWt [lane 3] and YCpSN2 [lane 5]) resulted in three additional products (132, 300, and 330 nucleotides long), and the splicing of IVS2 (YCpWt [lane 3] and YCpSN1 [lane 4]) resulted in one additional product 180 nucleotides long. These RNAs were not produced in YCpSN1-SN2.

The constructs bearing the *MATa1* gene lacking IVS1 and with point substitutions in the TACTAAC sequences of IVS2 (YCpSN10-26, YCpSN10-32, and YCpSN10-40) yielded mRNA transcripts that corresponded to the parent species of 441 nucleotides (Fig. 3B; data for YCpSN10-32 not shown). No RNA corresponding to the mature spliced message was observed, (Fig. 3B), indicating that splicing was completely disrupted. A transcript corresponding to 180 nucleotides for RNA from YCpSN10-26 (lane 1) and YCpSN10-40 (lane 3) was present at a higher level than was previously seen in the deletion series of constructs (Fig. 3A, lane 4). This transcript was absent in the RNA from YCpSN25-13, which has a point substitution in TACTAAC sequence of IVS1 and a deletion of IVS2 (lane 2). This transcript could correspond to a product with the lariat structure of IVS2 and exon 3 if the mutation were defective in 3′ excision. However, the primer extension analysis revealed a large amount of parent transcript. Hence, the putative **a**1′ protein could be produced.

The disruption of splicing of IVS1 with a point substitution in the internal conserved sequence in a gene lacking IVS2 (YCpSN25-13) resulted in a construct that partially repressed expression of the *HO::lacZ* fusion and allowed diploid cells to sporulate. The primer extension analysis revealed that the parent transcript was the only major transcript detectable (Fig. 3B). On longer exposure of the autoradiogram, a transcript corresponding to the mature message was detected (results not shown), suggesting that the point substitution was leaky. Therefore, it is possible that a fraction of the normal level of **a**1 protein is able to allow the cells to sporulate. There is the possibility of translation of IVS1 and readthrough of the amber codon present 8 nucleotides upstream of the internal conserved sequence of IVS1, resulting in a polypeptide with an insertion of 18 amino acid residues 35 amino acids from the N-terminal of the **a**1 protein sequence (Fig. 1). Interestingly, this construct showed no detectable levels of the RNA products 300 and 330 nucleotides in length.

DISCUSSION

We have addressed the question of whether both putative products of the *MATa1* gene, which arise through differential splicing of the second intron, are required for in vivo repression of haploid-specific functions. A series of constructs was generated by using oligonucleotide-directed mutagenesis that allowed us to evaluate the contribution of each of the polypeptides with respect to the ability to repress the expression of an *HO::lacZ* gene fusion and to allow diploid cells to undergo sporulation. The results of this analysis indicate that repression of transcription of the *HO::lacZ* fusion occurs efficiently in constructs in which both introns have been removed, thus ruling out the possibility of the involvement of polypeptide **a**1′ in repressing haploid functions. The same conclusions were reached when point mutations were introduced into the internal conserved sequence of IVS2 in a construct with a deletion of IVS1. This procedure resulted in disruption of splicing of IVS2 and hence the preferential presence of polypeptide **a**1′. No repression of β-galactosidase activity was observed, suggesting that **a**1′ cannot restore **a**1 functions. We also isolated mRNA from strains bearing these constructs to identify the transcripts and RNA-processing products of the *MATa1* gene by primer extension. Our observations clearly show that the constructs in the deletion series (YCpSN1, YCpSN2, and YCpSN1-SN2) produce transcripts, corresponding to unspliced IVS2, at higher levels in comparison with transcript levels with the unspliced first intron. The possibility therefore exists for a polypeptide (**a**1′) terminating within IVS2, as has been indicated previously (23). A construct of *MATa1* bearing a single point substitution in the internal conserved sequence of IVS2, which results in formation of mRNA for polypeptide **a**1′, is defective in repression of the fusion gene. This observation indicates that splicing of IVS2 and the amino acid residues beyond this intervening sequence are important for correct functioning of the **a**1 protein. Quantitative assessment of mating efficiency of a *MATα* strain carrying each of the *MATa1* constructs with a tester **a** strain also supports these observations and shows a block in mating (due to a functional **a**1 arising from YCpSN1, YCpSN2, and YCpSN1-SN2) or complete mating (due to a nonfunctional **a**1 arising from YCpSN10-26, YCpSN10-32, and YCpSN10-40). The exception is YCpSN25-13, which is unable to block mating, suggesting a nonfunctional or insufficient level of **a**1 polypeptide.

Although these results clearly point toward **a**1 as the functional polypeptide for repression of haploid functions, because of the limitations of the assays used for **a**1/α2 function, we do not rule out the possibility of a function for **a**1′. **a**1′/α2 may participate in a secondary pathway in the sporulation process, and the inability to form **a**1′ may affect the rate of sporulation but not the overall efficiency of the process.

The C-terminal portion of the **a**1 protein is predicted from sequence comparison analysis to contain the procaryote-type DNA-binding domain and also shares identity with the homeobox domain sequences of higher eucaryotes (20, 35). The specific requirement for the polypeptide segment encoded by the third exon of *MATa1* suggests that this region is important for function, perhaps in DNA binding. The homology to the homeobox domain proteins at the C terminus of the **a**1 protein extends 7 amino acid residues beyond the putative second helix of the helix-turn-helix motif. We are currently addressing the functional significance of the proteins.

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