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AAR2, a Gene for Splicing Pre-mRNA of the *MAT***a***1* Cistron in Cell Type Control of *Saccharomyces cerevisiae*

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We have isolated a class of mutants, *aar2*, showing the α mating type due to a defect in a1-α2 repression but with α2 repression activity from a nonmater strain of *Saccharomyces cerevisiae* expressing both a and α mating-type information in duplicate. Cells of the *aar2* mutant and the *aar2* disruptant also show a growth defect. A DNA fragment complementing the *aar2* mutation contains an open reading frame consisting of 355 amino acid codons. Northern hybridization showed that cells of the *aar2* mutant and disruptant contained α*1* and α*2* transcripts of the *MAT*α gene (or *HML*α in *sir3* cells), but their a*1* transcript of *MAT*a** (or *HMR***a** in *sir3* cells) migrated more slowly than that of the wild-type cells on gel electrophoresis and gave a diffused band. Primer extension analysis showed that the *aar2* mutant and disruptant have a defect in splicing two short introns of the a*1* pre-mRNA but not in splicing pre-mRNA of *ACT1*. The α mating type, but not the slow-growing phenotype, of the *aar2* mutant was suppressed by introduction of an intronless *MAT***a***1* DNA. Thus, the *AAR2* gene is involved in splicing pre-mRNA of the a*1* cistron and other genes that are important for cell growth. The *AAR2* locus was mapped on chromosome II beside the *SSA3* locus, with a 276-bp space, but was not allelic to either *PRP5* or *PRP6*, which are both located on chromosome II and function in splicing pre-mRNA of *ACT1*.**

The cell types of *Saccharomyces cerevisiae* are determined by the codominant *MAT***a** and *MAT*α alleles at the mating-type locus on the right arm of chromosome III (15). Haploid *MAT***a** cells have the **a** mating type and can mate with cells of the α mating type. Conjugation of **a** and α cells produces **a**/α diploid cells of a third cell type, nonmater. These **a**/α diploid cells undergo meiosis and form asci. There are two additional loci for complete mating-type information, *HML* and *HMR*, on the same chromosome, but their expression is repressed by the *SIR* genes. In general, *HML* has α information and *HMR* has **a** information. The *MAT*α locus consists of two cistrons, α*1* and α*2*. The α1 protein is necessary for expression of α-specific genes, and the α2 protein represses expression of **a**-specific genes. The *MAT***a** locus also contains two cistrons, a*1* and a*2*, and the a*1* cistron contains two short introns (24). The **a**-specific genes are expressed constitutively in **a** cells, and no activator protein encoded by *MAT***a** is needed. In conjunction with the α2 protein, the a1 protein represses the α*1* cistron, haploid-specific genes, and the *RME1* gene, a negative regulator for sporulation-specific genes (25) (a1-α2 repression). No particular functions of the a*2* cistron have been detected.

In a previous report, we have described two classes of mutants showing defective a1-α2 repression but normal α2 repression of **a**-specific genes (13). One of the classes had mutations in the α2 protein (13), and the other had mutations in a gene designated *AAR1*, which was found to be identical with previously identified genes *TUP1*, *SFL2*, *FLK1*, *CYC9*, *UMR7*, *AMM1*, and *AER2* (28). During isolation of similar mutants defective in a1-α2 repression but functional in α2 repression, we have isolated another class of mutants, *aar2*. This communication reports that the *AAR2* gene, located on chromosome II beside the *SSA3* locus (2), is involved in splicing pre-mRNA of the a*1* cistron and some of the other genes important for mitotic cell growth.

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MATERIALS AND METHODS

Microorganisms and plasmids. The *S. cerevisiae* strains used are listed in Table 1. All strains were heterothallic, as they had the *ho* genotype. These strains were constructed in our laboratory except for SPJ5.41, which was obtained from J. Abelson. *Escherichia coli* JA221 (5) and MV1184 (42) were used for manipulation of plasmid DNAs. Plasmids pUC118 and pUC119 (42) were used for preparation of single-stranded DNAs for DNA sequencing by the dideoxy termination method (36) with a helper phage, M13KO7 (42). A gene library of *S. cerevisiae*, YCp50 "CEN BANK" A (34), constructed by ligation of total genomic DNA partially digested with *Sau*3AI at the *Bam*HI site of YCp50 marked with the *URA3* gene, was obtained from the American Type Culture Collection (Rockville, Md.). Plasmid pYA301 (11) bearing the *ACT1* gene was provided by D. Gallwitz. Plasmid YCpSN1-SN2 (29) bearing intronless *MAT***a***1* DNA was obtained from M. Smith. Plasmids pYMC2 and pYMC3 were constructed by ligating a 3.3-kbp *Eco*RI-*Hin*dIII fragment bearing the full region of *MAT*α of plasmid 2.5 (obtained from J. B. Hicks) and that of *MAT***a** of plasmid DX (from J. B. Hicks), respectively, into the *Eco*RI-*Hin*dIII gap of YCp50. Plasmid pYMC5 bearing a 10.2-kbp *Sau*3AI fragment of the *AAR1* DNA inserted at the *Bam*HI site of YCp50 was obtained in a previous study (28). For construction of plasmid p530, used for preparation of an α*2* probe for Northern (RNA) hybridization, a 770-bp fragment of the region from nucleotide positions 753 to 1522 of the *MAT*α*2* gene (according to the numbering system of Astell et al. [1] for the *HML*α gene) was amplified by the polymerase chain reaction (PCR), using plasmid pKK24 (13) bearing the *MAT*α gene as a template. An oligonucleotide with the sequence 5′-CCCAAGCTTAGGAAGATAAGCAAGAAAAAATG-3′

TABLE 1. *S. cerevisiae* strains used

Strain	Mating type	Genotype
SH682	a	*MAT***a** *lys1 trp3 ura1 ura2 pho3 pho5*
SH683	α	*MATα lys1 trp3 ura1 ura2 pho3 pho5*
SH1263	α	*MATα ura3-52 leu2-3,112 trp1 his1-29 pho3-1 pho5-1*
SH1264	a	*MAT***a** *ura3-52*::[*ura3-52 HIS4-lacZ*][a] *leu2-3,112*
SH2645	Non	*MAT***a** *HMLα HMR***a** *sir3*::*LEU2*[b] *ura3 ade2-101*[ochre] *leu2-3,112*::[*LEU2 MFα1-PHO5*[c]] *trp1 his3Δ pho3-1 pho5-1*
SH2646	Non	*MAT***a** *HMLα HMR***a** *sir3*::*LEU2 ura3 trp1 ilv1 pho3-1 pho5-1 leu2-3,112*::[*LEU2 MFα1-PHO5*]
SH2780	Non	*MAT***a** *HMLα HMR***a** *sir3*::*LEU2 leu2-3,112*::[*LEU2 MFα1-PHO5*] *trp1*::[*TRP1 MATα*][d] *his3Δ pho3-1 pho5-1 ura3*
SH2780-m1	α	The *aar2-1* mutant of SH2780
SH2780-m2	α	The *aar2-2* mutant of SH2780
SH2925	Non	*MAT***a** *HMLα HMR***a** *sir3*::*LEU2 ura3 ilv1 pho3-1 pho5-1 leu2-3,112*::[*LEU2 MFα1-PHO5*] *trp1*::[*TRP1 MATα*]
SH2926	Non	A SH2780 + SH2925 fusant
SH2926-t	Non	An *AAR2/aar2*::*URA3* heterozygous diploid clone derived from SH2926
SH2926-t-1C	α	*MAT***a** *HMLα HMR***a** *sir3*::*LEU2 ura3 trp1*::[*TRP1 MATα*] *leu2-3,112*::[*LEU2 MFα1-PHO5*] *his3Δ ilv1 pho3-1 pho5-1 aar2*::*URA3*
SPJ 5.41	a	*MAT***a** *prp5-1*[ts] *ura3-52 leu2-3,112 his3 his7*
YP149	α	*MATα ura3-52 trp1Δ lys2-801 ade1 ade2-101*[ochre]

[a] *ura3-52*::[*ura3-52 HIS4-lacZ*] indicates a YIp plasmid bearing the *ura3-52* and *HIS4-lacZ* fusion DNA constructed and integrated at the *ura3-52* mutant locus by Lucchini et al. (21).

[b] A *sir3* locus disrupted by insertion of a *Bgl*II-*Xho*I *LEU2* fragment of *S. cerevisiae* (17).

[c] Active *PHO5* gene connected with the promoter of an α-specific gene, *MFα1* (18).

[d] *trp1*::[*TRP1 MATα*] represents integration of plasmid p443 with homology to the *TRP1* DNA into a *trp1* mutant locus. Plasmid p443 was constructed by ligation of a 4.3-kbp *Pvu*I-*Bam*HI fragment containing the *MATα* gene, prepared from plasmid 2.5, with a 4.6-kbp *Pvu*I-*Bgl*II fragment containing the *TRP1* gene of YRp7.

(corresponding to the sequence from 1500 to 1522 of *MATα2* with an additional *Hin*dIII recognition sequence and CCC at the 5′ end) was used as the forward primer, and a 5′-CCCG GATCCATCTATCAGTTACAAACATCTTA-3′ fragment (corresponding to the sequence from 753 to 775 with an additional *Bam*HI recognition sequence and CCC at the 5′ end) was used as the reverse primer. These oligonucleotides were synthesized chemically in a DNA synthesizer (Gene Assembler Plus; Pharmacia LKB, Uppsala, Sweden). The amplified product was digested with *Hin*dIII and *Bam*HI and cloned into the *Hin*dIII-*Bam*HI gap of pBluescript II KS$^+$ (Stratagene, La Jolla, Calif.). Plasmid p531, used for preparation of an **a***1* probe, was constructed similarly with a 521-bp DNA fragment bearing the nucleotide region from positions 1506 to 2132, but without the 106-bp intron sequence, of the *MAT***a***1* gene (according to the numbering system of Astell et al. [1]) obtained by amplification of the DNA by PCR. For PCR, plasmid YCpSN1-SN2 containing intronless *MAT***a***1* DNA (29) was used as a template, an oligonucleotide of the sequence 5′-CCCAAGCTTAGAAAA TCAAGAAGGACAACATG-3′ (nucleotide positions 1506 to 1528 of *MAT***a** with a *Hin*dIII recognition sequence and CCC at the 5′ end) was used as the forward primer, and another oligonucleotide, 5′-CCCGGATCCCATAATTATTC GTCAACCACTCTA-3′ (sequence from 2132 to 2109 of *MAT***a** with a *Bam*HI recognition sequence and CCC at the 5′ end), was used as the reverse primer. Plasmid YCp50 (33) was used as a low-copy-number vector, YIp5 (33) was used as an integrative vector, and YRp7 (33) was used for preparation of the *TRP1* fragment. The sources and structures of the other plasmids used are described in the text.

Media and genetic methods. YPAD and SD media, used for cultivation of *S. cerevisiae*, and LB medium, used for *E. coli* cells, were described previously (45). The genetic methods used for *S. cerevisiae* were as described previously (38). Hybridization of two *S. cerevisiae* strains of the same mating type and mating of nonmater strains were achieved by cell fusion of protoplasts (14). *S. cerevisiae* was transformed by the method of Ito et al. (16), and *E. coli* was transformed by the method of Morrison (26).

Biochemical methods. The methods for preparation and manipulation of DNAs and RNAs were as described previously (31, 38). Nucleotide sequences were determined by the dideoxy-chain termination method (36). For detection of mature and immature transcripts from *MAT***a***1* and *ACT1*, primer extension was performed as described by Domdey et al. (7). Oligonucleotides 5′-TAGATCTCATACGTTT-3′ (sequence from 2007 to 1992 of *MAT***a***1* [1]) and 5′-AACCGT TATCAATAACCAAA-3′ (from +346 to +327 of *ACT1* [10]) were synthesized in the DNA synthesizer as described above and phosphorylated at the 5′ ends with [γ-^{32}P]ATP, using a Megalabel kit (Takara Shuzo Co. Ltd., Kyoto, Japan). Reverse transcriptase (Rous-associated virus-2) was purchased from Takara Shuzo. Chromosomes of *S. cerevisiae* were separated by contour-clamped homogeneous electric field gel electrophoresis (4). Acid phosphatase (APase; EC 3.1.3.2) activity of *S. cerevisiae* colonies was detected by staining based on a diazo-coupling reaction (39).

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the DDBJ, EMBL, and GenBank data banks under accession number D90455.

RESULTS

Isolation and genetic characterization of *aar2* mutants. To detect a protein participating in cell type control other than those produced from the mating-type genes, we isolated mutants defective in **a**1-α2 repression. To facilitate isolation of such mutants, we constructed *S. cerevisiae* SH2780, which expressed **a** and α information in duplicate due to expression of the *MAT***a** gene, a *MATα* gene inserted at the *trp1* locus, and the *HMLα* and *HMR***a** loci resulting from disruption of *SIR3*. This strain was defective in genomic APase genes (*pho3-1* and *pho5-1*) and had an active *PHO5* gene under control of the promoter of an α-specific gene, *MFα1* (18). Because the cells have both **a** and α information, the α*1* cistron was repressed, and consequently no α-specific genes, including the *MFα1-PHO5* construct, were expressed. Hence, colonies of this strain were white (i.e., APase$^-$) when stained for APase activity. If mutants have a defect in **a**1-α2 repression while retaining α2 repression, they should have the α mating type and the colonies should stain red (APase$^+$).

Cells of strain SH2780 were subjected to ethyl methanesulfonate mutagenesis, plated on YPAD plates, and incubated at 30°C for 2 to 3 days. Of approximately 20,000 colonies that formed on the plates, 100 were pink or red, i.e.,

stained positively for APase activity. The mating of cells in these colonies was tested by cross-streaking with cells of standard strains SH682 and SH683 for mating types **a** and α, respectively. Eleven of them were found to have the α mating type, although the mating potencies of cells in individual clones differed. These mutants were then examined to determine whether the mutations occurred in *MAT***a**, *MAT*α, *AAR1*, or other genes by transforming them with pYMC3, pYMC2, and pYMC5, YCp50-based plasmids bearing *MAT***a**, *MAT*α, and *AAR1* DNAs, respectively, and by staining the transformants for APase activity. The transformants derived from 2 of the 11 isolates, designated SH2780-m1 and SH2780-m2, still developed a red color, whereas the mutant phenotypes of the other 9 clones were suppressed by one of these plasmids (data not shown). These two mutants had the α mating type, although their mating potencies were less than that of wild-type cells, and showed slow growth in YPAD medium.

Cells of SH2780-m1 and -m2 were each fused with cells of the wild-type strain SH2646 (*MAT***a** *HML*α *HMR***a** *sir3*:: *LEU2 MF*α*1-PHO5*). The resultant hybrids exhibited the nonmating phenotype and no APase activity. Thus, the mutations were recessive. SH2780-m1–SH2646 fusants were sporulated, and asci were dissected. The mating types and APase activities of nine four-spored asci were tested. Results showed 2α:2 nonmater and 2 APase$^+$:2 APase$^-$ segregation in all asci, and all of the spore clones with the α mating type showed the APase$^+$ phenotype. The slow-growing phenotype also cosegregated with the α and APase$^+$ phenotypes. These results clearly indicate that a single nuclear mutation confers these pleiotropic phenotypes.

To identify the mutations in SH2780-m1 and -m2, we selected a tetrad segregant from the SH2780-m1–SH2646 fusant showing the α mating type and APase$^+$ phenotype and complementary His$^+$ *ilv1* markers. This segregant was fused with SH2780-m2 (*his3* Ilv$^+$). The His$^+$ Ilv$^+$ fusant exhibited the α mating type and was APase$^+$, indicating that these two mutations did not complement each other. Thus, we designated the mutation in SH2780-m1 as *aar2-1* and that in SH2780-m2 as *aar2-2*.

Cloning of the *AAR2* gene. For cloning of *AAR2*, the original *aar2-1* mutant was transformed with the YCp50-based *S. cerevisiae* gene bank, YCp50 "CEN BANK" A. Four Ura$^+$ transformants showing normal cell growth were nonmating type and APase$^-$, indicating that the *aar2* mutant phenotypes were suppressed simultaneously. DNA was prepared from one of these transformants and purified in *E. coli*. A plasmid thus obtained conferred the nonmating, APase$^-$, and normal cell growth phenotypes on both of the original *aar2* mutants. This plasmid, p508 (Fig. 1), had an insert of a 6.0-kbp *Sau*3AI fragment at the *Bam*HI site of YCp50.

We confirmed that the cloned DNA fragment carried the *AAR2* gene by its site-directed integration into an *S. cerevisiae* chromosome. An 8.0-kbp *Aat*II$_{(2)}$-*Sma*I fragment of p508 was subcloned into the *Aat*II-*Sma*I gap of YIp5. The resultant plasmid, p509 (Fig. 1), was linearized by restriction at the unique *Hin*dIII site in the cloned fragment and used to transform SH2780-m1 (*aar2-1 ura3*). One of the Ura$^+$ transformants showing the nonmating, APase$^-$, and normal cell growth phenotypes was fused with SH2646 (*MAT***a** *HML*α *HMR***a** *sir3*::*LEU2 AAR2*$^+$), and the diploid was subjected to tetrad analysis. All four tetrad clones in 14 asci dissected showed nonmater, APase$^-$, and normal cell growth phenotypes. Hence, p509 DNA is integrated at the *aar2* locus and the cloned DNA carries the *AAR2* gene. Since a 2.0-kbp *Aat*II$_{(2)}$-*Nru*I$_{(1)}$ fragment of *AAR2* prepared from p508 could hybridize with the band of chromosome II on a contour-clamped homogeneous electric field gel applied with a chromosome sample of strain YP149 (data not shown), the *AAR2* gene is on chromosome II.

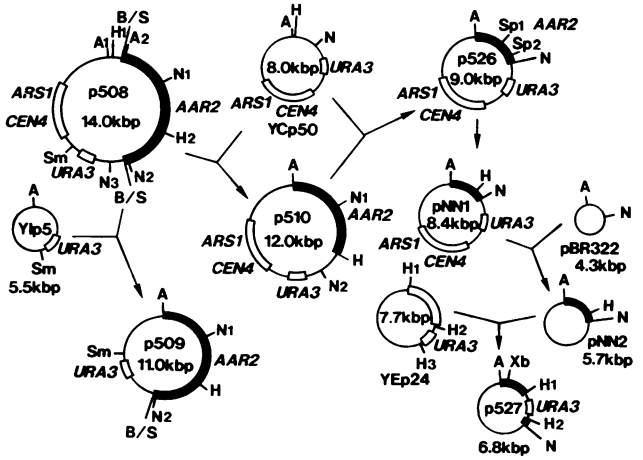

FIG. 1. Structures and strategy for construction of plasmids bearing the *AAR2* DNA. The procedures for construction or derivation of plasmids p508, p509, and p526 are described in the text. p510 was constructed by inserting a 4.0-kbp *Aat*II$_{(2)}$-*Hin*dIII$_{(2)}$ fragment from p508 into the *Aat*II-*Hin*dIII gap of YCp50. p527 was constructed via two intermediate plasmids, pNN1 and pNN2. pNN1 was obtained by eliminating a 0.58-kbp *Sph*I$_{(1)}$-*Sph*I$_{(2)}$ region from p526 and recircularized after the *Sph*I ends were changed to *Hin*dIII sites by digesting the overhanging 3′ end to a blunt end with T4 DNA polymerase and connecting an 8-bp *Hin*dIII linker (Takara Shuzo). Then a 1.5-kbp *Aat*II-*Nru*I fragment of pNN1 was ligated with a 3.3-kbp *Aat*II-*Nru*I fragment of pBR322 to give plasmid pNN2. p527 was obtained by inserting a 1.1-kbp *Hin*dIII$_{(2)}$-*Hin*dIII$_{(3)}$ fragment bearing the *URA3* gene from YEp24 into the *Hin*dIII site of pNN2. Restriction sites: A, *Aat*II; H, *Hin*dIII; N, *Nru*I; Sm, *Sma*I; Sp, *Sph*I; Xb, *Xba*I. B/S is the junction site of *Bam*HI and *Sau*3AI. Two or more identical restriction sites are distinguished by suffixes. Closed boxes indicate the *AAR2* DNA; open boxes represent various DNA fragments of *S. cerevisiae* chromosomes other than the *AAR2* DNA. The thin lines indicate the DNA fragment originating from pBR322.

Nucleotide sequence of the *AAR2* gene. The *AAR2* region was delimited to a 2.0-kbp *Aat*II-*Nru*I$_{(1)}$ region of the 6.0-kbp *Sau*3AI *AAR2* fragment, because this 2.0-kbp fragment cloned into YCp50 (the resultant plasmid is p526; Fig. 1) was able to suppress the *aar2* mutation of SH2780-m1 (Fig. 2). We sequenced a 2.6-kbp *Aat*II-*Mnl*I region containing this 2.0-kbp region. A single open reading frame (ORF) consisting of 1,065 bp was found in the fragment (Fig. 3). The predicted Aar2 protein consists of 355 amino acid residues with a molecular size of 41 kDa.

No significant similarities of this protein with any known proteins were detected in searches with the nucleotide data base of the European Molecular Biology Laboratory (release 13.0; December 1990) by using the GENETYX program (Software Development Co., Ltd., Tokyo, Japan). However, during the homology search, we found the 5′ end of the *SSA3* gene encoding heat shock protein HSP70 (2) 276 bp downstream from the C-terminal codon of *AAR2* (Fig. 2 and 3). Boorstein and Craig (2) reported the presence of part of an unknown ORF consisting of at least 308 amino acid codons in a cloned *SSA3* fragment. This sequence completely conformed with the corresponding region of the *AAR2* gene.

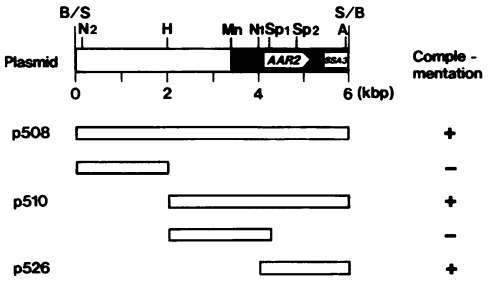

FIG. 2. Restriction map of the *AAR2* DNA and delimitation of the functional region. Construction and derivations of p508, p510, and p526 are described in the text and the legend to Fig. 1. Complementation of the *aar2* mutation was detected by testing the ability to complement the slow cell growth, α mating type, and APase$^+$ phenotypes of transformants with the respective plasmids in cells of strain SH2780-m1 (*aar2-1 ura3 MFα1-PHO5*). The open and closed boxes at the top represent the cloned yeast DNA. Nucleotide sequences were determined for the closed region as indicated by the open arrow and open box; the open arrow with *AAR2* indicates the approximate position and direction of the ORF of *AAR2*, and the open box with *SSA3* indicates the N-terminal coding region of the *SSA3* gene (2). + and − represent ability and inability, respectively, to complement the *aar2* mutant phenotypes. Mn represents the restriction site of *Mnl*I; other abbreviations for restriction sites are as in Fig. 1.

An A-rich region is present from nucleotide positions −349 to −321 in the *AAR2* DNA in the 5′ upstream region of the ORF relative to the translational initiation codon, and putative TATA boxes are at nucleotide positions −167 to −163 and −232 and −228. However, no sequences homologous to the terminator sequence proposed by Zaret and Sherman (46) were found in the 3′ noncoding region. It is noteworthy that one perfect match to the pheromone-responsive element (5′-TGAAACA-3′), which is known to be a binding site of Ste12 protein in the upstream region of pheromone-inducible genes (6, 9), was found at nucleotide positions −398 to −392, and three copies of inverted sequences of this element with imperfect (six of seven) matches were found at −98 to −92, −294 to −288, and −416 to −410. In the predicted Aar2 protein, we found a region homologous to the leucine zipper structure (19) from amino acid positions 261 to 282 and a segment rich in acidic amino acid residues from 332 to 341.

Disruption of *AAR2* confers a growth defect. To investigate its function, we disrupted the *AAR2* gene by the procedure of one-step gene disruption (35). A 1.9-kbp *Nru*I-*Xba*I fragment of p527 (Fig. 1) bearing the *AAR2* DNA disrupted with an insertion of a 1.1-kbp *URA3* DNA was prepared and used to transform cells of a wild-type diploid strain, SH2926 (homozygous for the *MAT***a** *HMLα HMR***a** *sir3*::*LEU2 ura3* [*TRP1 MATα*] [*LEU2 MFα1-PHO5*] *AAR2*$^+$ genotype). One of the Ura$^+$ transformants, SH2926-t, isolated at random,

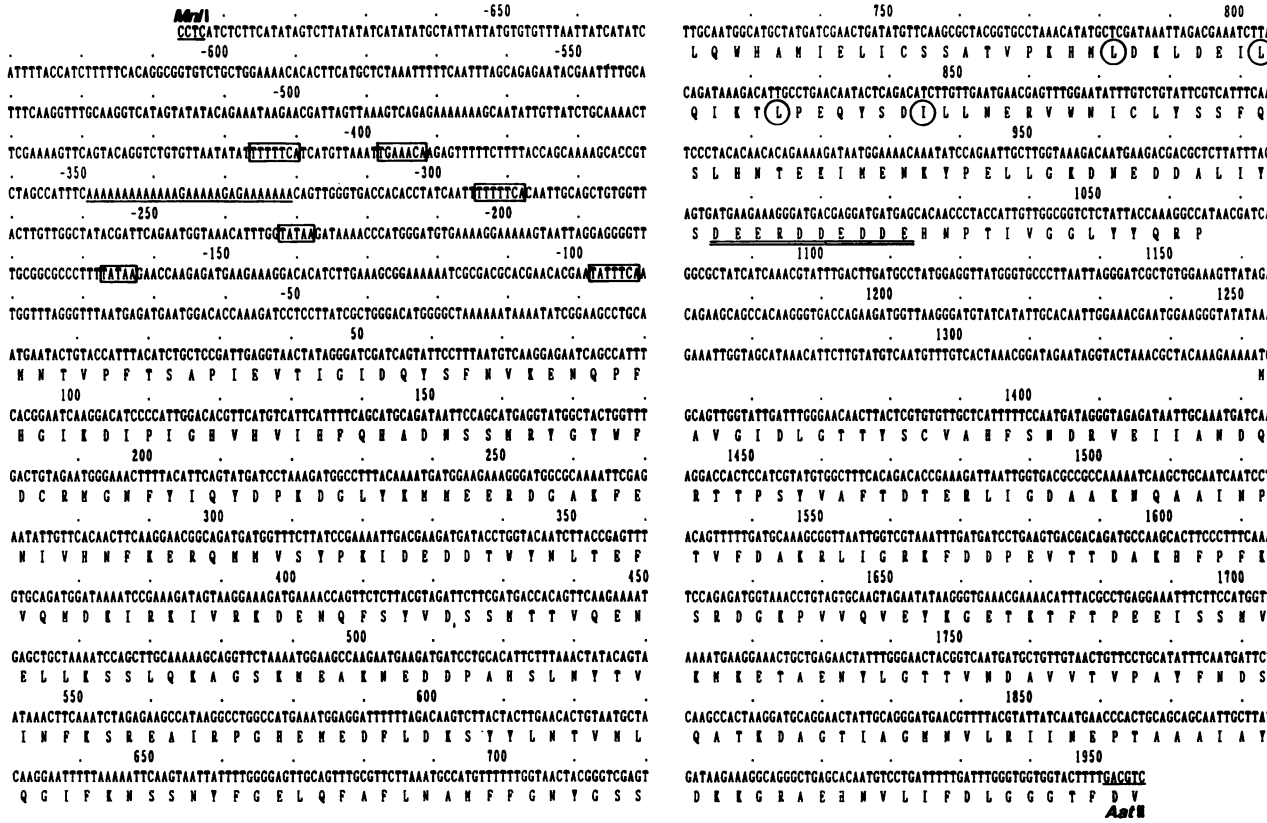

FIG. 3. Nucleotide sequence of the *AAR2* gene and deduced amino acid sequence. The ORF start site at +1 is that of *AAR2*, and the ORF start site at 1342 is that of *SSA3*. The underline in the 5′ upstream region of the *AAR2* ORF indicates an A-rich region. Putative TATA and pheromone-responsive (5′-TGAAACA-3′) elements are enclosed in boxes. The four encircled amino acid residues in the *AAR2* ORF represent the leucine (or isoleucine) residues analogous to the leucine zipper structure. A peptide segment rich in acidic amino acid residues is doubly underlined.

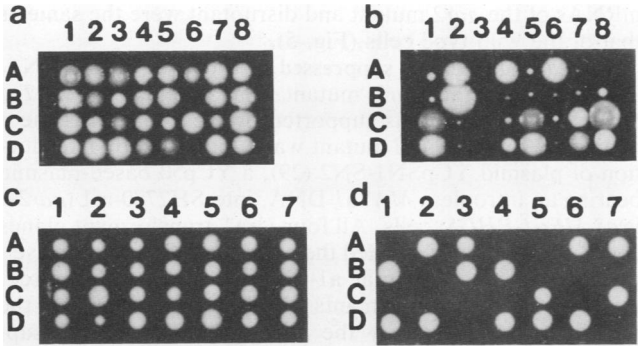

FIG. 4. Growth properties of tetrad spores having the *aar2-1* mutation or the *aar2*::*URA3* disruptant allele. (a and b) Diploids constructed by protoplast fusions of SH2780 plus SH2645 (*AAR2*$^+$ *ADE2*$^+$/*AAR2*$^+$ *ade2*; a) and SH2780-ml plus SH2645 (*aar2-1* *ADE2*$^+$/*AAR2*$^+$ *ade2*; b) were sporulated, dissected on a YPAD plate, and incubated at 30°C for 3 (a) or 5 (b) days. (c and d) Similar dissection plates of diploid strain SH2926 (*AAR2*$^+$/*AAR2*$^+$; c) and its *AAR2*$^+$/*aar2*::*URA3* derivative (SH2926-t; d) were incubated at 30°C for 3 (c) or 5 (d) days. Four spores (rows A to D) from individual tetrads (lanes 1 to 8 or 7) were arrayed in columns.

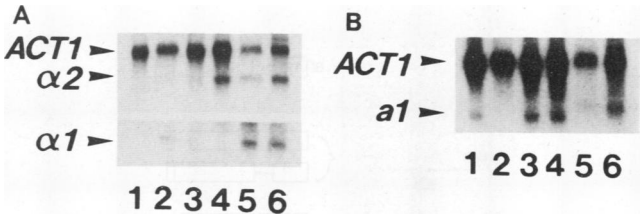

FIG. 5. Northern analysis of $\alpha 1$, $\alpha 2$, and **a***1* transcription levels in the *aar2* mutant and the *aar2*::*URA3* disruptant. Poly(A)$^+$ RNAs were prepared from cells of strains SH1264 (*MAT***a** *AAR2*$^+$; lanes 1), SH1263 (*MAT*α *AAR2*$^+$; lanes 2), SH2645 (*MAT***a** *HML*α *HMR***a** *sir3*::*LEU2* *AAR2*$^+$; lanes 3), SH2780 (*MAT***a** *HML*α *HMR***a** *sir3*::*LEU2* [*TRP1* *MAT*α] *AAR2*$^+$; lanes 4), SH2780-ml (*MAT***a** *HML*α *HMR***a** *sir3*::*LEU2* [*TRP1* *MAT*α] *aar2-1*; lanes 5), and SH2926-t-1C (*MAT***a** *HML*α *HMR***a** *sir3*::*LEU2* [*TRP1* *MAT*α] *aar2*::*URA3*; lanes 6). Samples of 1 μg of poly(A)$^+$ RNA were put into slots of a formaldehyde-agarose gel (1.5%). (A) A ^{32}P-labeled 1.1-kbp *Eco*RV fragment bearing the *MAT*α*1* gene prepared from plasmid 2.5 was used to detect the $\alpha 1$ transcript. A 0.8-kbp *Bam*HI-*Hin*dIII fragment prepared from p530 bearing the *MAT*α*2* gene was used to detect the $\alpha 2$ transcript. (B) A 0.6-kbp *Bam*HI-*Hin*dIII fragment encoding *MAT***a***1* prepared from p531 was used as a probe to detect the **a***1* transcript. A 1.1-kbp *Xho*I-*Hin*dIII fragment encoding the *ACT1* gene of *S. cerevisiae* prepared from pYA301 (9) was used as the probe for an internal marker. The specific activity of each probe was adjusted to 10^8 cpm/μg of DNA.

was subjected to tetrad analysis. On examination of 19 asci, we observed an apparent ratio of 2 visible:2 invisible colonies on dissection plates incubated at 30°C for 5 days after ascus dissection, whereas the in vivo *aar2* mutant could, in general, develop minute visible colonies (Fig. 4). All of the visible colonies showed the Ura$^-$ phenotype. However, by microscopy we observed minute colonies at the remaining two spots for each tetrad where no macroscopic colonies developed. This fact indicates that these spore clones could germinate and achieve some growth. After incubation of the dissection plates for 10 days at 30°C, we observed two minute colonies at the positions of two of the microscopic colonies but not at positions of the others. Cells from these two minute colonies showed the α mating type and APase$^+$ phenotypes, like *aar2* mutants, but exhibited more severely reduced growth than did the original *aar2* mutants. The Ura$^+$ phenotype of the minute clones suggested that they might have a disrupted *AAR2* locus. This was confirmed by Southern hybridization analysis using an *Eco*RI digest of genomic DNA of one of the minute clones, SH2926-t-1C (data not shown). Unlike the *aar2-1* and *aar2-2* mutants, cells of these two disruptants could not grow at 37°C. These findings suggest that disruption of the genomic *AAR2* gene resulted in a severe defect in cell growth, such that most cells were inviable even at 25°C, and all of the cells were completely inviable at 37°C. Another possibility is that disruption of *AAR2* was in fact lethal and that the two minute clones were those of cells with a secondary mutation that allowed growth of the gene disruptants.

Defect of a1-α2 repression in the *aar2* mutants. To determine why the *aar2* mutants showed the α mating type, we analyzed **a***1*, $\alpha 1$, and $\alpha 2$ transcripts of SH2780-ml (*aar2-1*) and SH2926-t-1C (*aar2*::*URA3* disruptant) by Northern hybridization. The *aar2* mutant (Fig. 5A, lane 5) and disruptant (lane 6) with expressed *MAT***a**, *MAT*α, and *HML*α contained $\alpha 1$ mRNA (ca. 770 bases [1]), like the α *AAR2*$^+$ wild-type cells (lane 2) but not **a** *AAR2*$^+$ wild-type cells (lane 1). In the *AAR2*$^+$ cells with expressed *MAT***a** and *MAT*α and/or *HML*α, $\alpha 1$ transcription was almost repressed (Fig. 5A; lanes 3 and 4). The $\alpha 2$ transcript (ca. 770 bases [1]) was detected in all but the **a** cells. These results indicate that the α mating type and APase production from the *MF*α*1-PHO5* fusion gene in the *aar2* mutants might be caused by a defect in **a**1-α2 repression.

The **a***1* transcript (ca. 450 bases after splicing of the introns [1, 24]) was detected in all but the α-cell mRNA samples (Fig. 5B). However, we noticed that the **a***1* transcript in the *aar2* mutant (lane 5) and the *aar2* disruptant (lane 6) migrated more slowly than in the *AAR2*$^+$ cells and showed a diffused band. Since the *MAT***a***1* cistron contains two short introns (24), 54 and 52 nucleotides in length, and since unspliced pre-mRNA of *MAT***a***1* has no function (29), the defect in **a**1-α2 repression in the *aar2* mutants is probably caused by inability to splice the *MAT***a***1* transcript properly.

The *aar2* mutant is defective in splicing of *MAT*a***1* mRNA.** To investigate the splicing of the **a***1* transcript, we carried out primer extension analysis with poly(A)$^+$ RNAs from cells of the *aar2-1* mutant and disruptant by using an oligonucleotide complementary to the 3′ end of the **a***1* message. The wild-type cells showed three major bands corresponding to unspliced transcript, mature message, and the transcript having one of the two introns unspliced (Fig. 6). The intensities of the bands indicated that the mature message and transcripts containing one unspliced intron were present in approximately equal amounts, whereas the unspliced transcript was the minor species. In contrast, the *aar2* mutant and disruptant contained unspliced transcript and that having one unspliced intron in almost equal amounts, but the mature message was detected only in trace amount. These results indicated that the *aar2* mutation confers severely reduced splicing efficiency of *MAT***a***1* mRNA.

***ACT1* mRNA is spliced in the *aar2* mutant.** We used the *ACT1* transcript as an internal control in Northern analysis of the messages from mating-type genes (Fig. 5). The pre-mRNA of *ACT1* is reported to be about 1,250 bases (10) and to contain a 304-base (10) or 309-base (30) intron start at +11 relative to the ATG codon (10, 30). For determination of whether the pre-mRNA of *ACT1* is spliced in the *aar2*

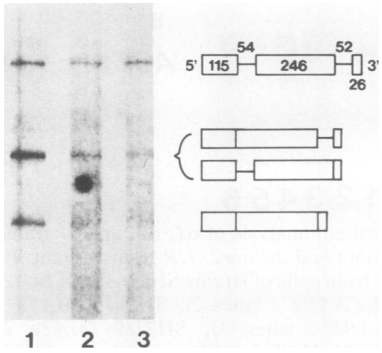

FIG. 6. Primer extension analysis of **a***1* pre-mRNA in the *aar2* mutant. Poly(A)$^+$ RNAs were prepared from cells of strain SH2780 (*AAR2*$^+$; lane 1), SH2780-m1 (*aar2-1*; lane 2), and SH2926-t-1C (*aar2*::*URA3*; lane 3) and hybridized with the ^{32}P-labeled oligonucleotide described in Materials and Methods by the procedure described by Domdey et al. (7). Primer extension products were purified and electrophoresed on a polyacrylamide sequencing gel containing 7 M urea. Suggested structures of the **a***1* transcripts are shown on the right. Open boxes and lines are exons and introns, respectively. The numbers are the lengths of the exons and introns in bases (29).

mutants, primer extension analysis was carried out for the *AAR2*$^+$, *aar2-1* mutant, and *aar2* disruptant used for detection of the *MAT***a***1* message, using an oligonucleotide complementary to the *ACT1* message near the 5′ end of the intron. Since the 5′ end of the *ACT1* transcript is at a site -140 ± 30 (30), the reaction product by primer extension should have 180 ± 30 bases. All of the test strains showed only one band of approximately 160 bases, while a few-bases-longer message was found in the *aar2* disruptant than in the others for an unknown reason (Fig. 7). This finding indicates that the pre-mRNA of *ACT1* is spliced properly in the *aar2* mutant and is in accord with the observation by Northern hybridization that the mobilities of the *ACT1* mRNAs of the *aar2* mutant and disruptant were the same as that of the wild-type cells (Fig. 5).

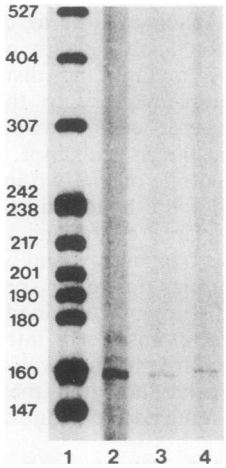

FIG. 7. Splicing of *ACT1* pre-mRNA in *aar2* mutants. Total RNAs were prepared from the cells of strain SH2780 (*AAR2*$^+$; lane 2), SH2780-m1 (*aar2-1*; lane 3), and SH2926-t-1C (*aar2*::*URA3*; lane 4) and hybridized with a ^{32}P-labeled oligonucleotide described in Materials and Methods under the conditions used for detection of **a***1* transcripts (Fig. 6). *Hap*II-digested fragments (in bases) of pBR322 DNA were run in parallel (lane 1) as size markers.

The *aar2* mutation is suppressed by intronless *MAT*a DNA. The idea that the *aar2* mutant has a defect in *MAT***a***1* pre-mRNA splicing was supported by the finding that the α mating type of the *aar2* mutant was suppressed by introduction of plasmid YCpSN1-SN2 (29), a YCp50 based-plasmid bearing an intronless *MAT***a***1* DNA, into SH2780-m1 (*aar2-1 ura3 MF*α*1-PHO5*) cells. All four Ura$^+$ transformant clones isolated at random exhibited the nonmating type and APase$^-$ phenotype, indicating that **a**1-α2 repression was restored. However, as the transformants still showed slow growth, the growth defect caused by the *aar2* mutation was not suppressed by the intronless *MAT***a***1* DNA. When plasmid YCpSN1-SN2 was cured from the transformants, the cells again showed the same phenotype as did the original *aar2* mutant.

The *aar2* mutation is not allelic to *prp5* and *prp6*. We considered whether *aar2* is allelic to either one of two mutations, *prp5* (formerly *rna5* [22]) and *prp6* (*rna6*) (43), because these mutations, which confer a defect in splicing of mRNA (22), both mapped on chromosome II (27). The *PRP6* gene has recently been sequenced (20), and its sequence clearly differs from that of the *AAR2* gene. The *AAR2* gene also differs from *PRP5* as shown in an allelism test between the *aar2* and *prp5* mutations by crossing strains SH2926-t-1C (α *aar2* disruptant) and SPJ 5.14 (**a** *prp5*; unable to grow at 37°C). The diploid cells showed normal growth at 37°C.

DISCUSSION

We have identified a new gene, *AAR2*, that is necessary for **a**1-α2 repression and for vegetative cell growth. The *aar2* mutant cells have the α mating type because they have a defect in **a**1-α2 repression but functional α2 repression. Primer extension experiments indicated that the *aar2* mutant has a defect in splicing of the *MAT***a***1* transcript (Fig. 6), while the mutation does not affect *ACT1* splicing (Fig. 7). This was supported by the finding that introduction of intronless *MAT***a***1* DNA suppressed the α mating type and APase$^+$ phenotypes with the *PHO5* gene connected downstream of the *MF*α*1* promoter in the *aar2* mutant cells but did not suppress their phenotype of slow growth. We conclude that *AAR2* is involved in splicing pre-mRNA of *MAT***a***1* and that of some of the unidentified genes that are important for cell growth, but not in splicing pre-mRNA of *ACT1*.

Several *PRP* genes were known to be involved in pre-mRNA splicing in *S. cerevisiae* (for a review, see reference 44); and *PRP2*, at least, has been shown to be directly involved in splicing the **a***1* pre-mRNA (24). The *ACT1* gene, one of the intron-containing genes in *S. cerevisiae* that is essential for cell growth, has often been used in studies on the mechanism of RNA splicing by *PRP* genes, and a temperature-sensitive *prp2* mutant has been found not to splice *ACT1* mRNA at the nonpermissive temperature (43). However, the *ACT1* transcript, which we happened to use as an internal control in Northern analysis of α*1*, α*2*, and **a***1* transcripts (Fig. 5), was spliced normally in the *aar2* mutant (Fig. 7). The results consequently indicate that the *AAR2* gene is not involved in splicing *ACT1* mRNA and may differ from reported *PRP* genes, including *PRP2*, in specificity for splicing pre-mRNA species.

Splicing of RNAs occurs in large complexes, called spliceosomes, consisting of various proteins and small nuclear RNAs (12, 32). If the Aar2 protein is a component of spliceosomes, it must enter the nucleus because splicing

processes occur in the nucleus. No possible signals for nuclear localization were found in the amino acid sequence of the putative Aar2 protein. The protein does, however, have a sequence analogous to that of a leucine zipper structure, which has been implicated in the homo- and heterodimerization processes of some proteins (37). Hence, the Aar2 protein may interact in the cytoplasm with a component of spliceosomes via the leucine zipper and then be transported into the nucleus. Possible candidate proteins are Prp6 and Prp9, which commit pre-mRNA to the splicing pathway, as their predicted amino acid sequences both contain a leucine zipper structure (20).

We noticed that the upstream region of the *AAR2* gene contains one copy of a pheromone-responsive element (5′-TGAAACA-3′ [Fig. 3]) (and three copies of the same motif with six-of-seven-base identity but in the reverse direction) which is often found in the upstream region of pheromone-inducible genes such as *FAR1* (3), *FUS1* (23, 40, 41), and *FUS3* (8). Furthermore, we could not detect a transcript of *AAR2* in cells in the logarithmic growth phase (data not shown), suggesting that the level of the *AAR2* transcript is very low in the absence of induction with an appropriate mating pheromone. The interesting problems of transcriptional regulation of *AAR2* and its splicing specificities toward *MAT***a***1* and various other intron-containing genes remain to be elucidated.

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