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A Functional Prepro-α-Factor Gene in *Saccharomyces* Yeasts Can Contain Three, Four, or Five Repeats of the Mature Pheromone Sequence

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The chromosomal region containing a structural gene for the mating pheromone precursor prepro-α-factor was examined in a variety of *Saccharomyces* yeasts by using a cloned putative prepro-α-factor gene of *Saccharomyces cerevisiae* as the probe. Analysis by restriction endonuclease digestion and Southern blot hybridization indicated that the physical arrangement of this region is highly conserved in all the *Saccharomyces* species analyzed, but displays length polymorphisms of limited size (50 to 60 base pairs). The observed polymorphisms were shown to be due solely to differences in the number of tandemly arranged spacer peptide/pheromone units within the coding sequence of these genes. Analysis of polyadenylated RNA indicated that these genes specified RNA transcripts and that these RNA molecules could be translated in vitro into prepro-α-factor polypeptides immunoprecipitable with anti-α-factor antibodies. The sizes of both the mRNAs and the proteins synthesized from them reflected exactly the differences observed in the lengths of the genes. These findings demonstrate conclusively that the putative prepro-α-factor DNA cloned from *S. cerevisiae*, as well as the sequences detected in the other *Saccharomyces* species, are indeed expressed and functional genes, and suggest that proper proteolytic processing of prepro-α-factor is unaffected by the number of pheromone repeats encoded within this precursor protein.

Each of the haploid cell types (**a** and α) of the yeast *Saccharomyces cerevisiae* secretes a specific oligopeptide mating pheromone (reviewed in reference 38). The peptide released by cells of the α mating type is called α-factor. This molecule is only 13 residues long; however, evidence has been obtained that indicates α-factor is produced by processing of a larger precursor polypeptide. We have demonstrated that strains carrying a *ste13* mutation (32) produce forms of α-factor which are larger because these molecules have not been matured completely at their amino terminal ends due to a deficiency of a membrane-bound processing enzyme, dipeptidyl aminopeptidase A (15). The block of additional residues not removed from α-factor molecules in *ste13* mutants corresponds precisely to portions of the amino acid sequence of a potential 165-residue-long precursor (prepro-α-factor) deduced from the nucleotide sequence of a clone of the putative structural gene (18). The proposed prepro-α-factor precursor of *S. cerevisiae* has a canonical "signal" sequence of 20 or so mainly hydrophobic residues, an additional leader region of about 60 hydrophilic amino acids, and four identical tandem repeats of the mature pheromone sequence (13 amino acids) separated by short spacer peptides (six to eight residues), each of identical or very similar amino acid sequence. The extra amino acids in the α-factor-related peptides produced by *ste13* mutants correspond to these spacer regions. Hence, the prepro-α-factor molecule is apparently a polyprotein precursor.

Although the properties of the misprocessed peptides accumulated by *ste13* mutants largely corroborate the existence of the larger progenitor molecule, we sought independent means to confirm the structure of the prepro-α-factor precursor and to determine whether such a protein molecule indeed represents the primary translation product of the gene identified by recombinant DNA methodology. We reasoned that if the α pheromone is essential for the mating process in *Saccharomyces* yeasts, then the physical arrangement of its structural gene, the prepro-α-factor protein therein encoded, and perhaps even its flanking chromosomal segments should all be conserved to a high degree. Therefore, as one approach to these questions, the structure of

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the α-factor gene, the existence of complementary transcripts, and the coding capacity of these same mRNAs were examined in several different *S. cerevisiae* strains and in a variety of other related *Saccharomyces* species.

MATERIALS AND METHODS

Yeast strains and culture conditions. The yeast strains used in this study are listed in Table 1. Strain AB723 was constructed by mating strain 277 (a derivative of the original prototrophic homothallic [*HO*] *Saccharomyces oviformis* strain 279), which carried appropriate markers and had an **a** mating phenotype, with XT1172-S245C (*ho*), an α haploid. The resulting diploid (*HO/ho*) was sporulated and the asci produced were dissected. Spores were scored for stable α character by crossing to mating type tester strains (DC14 *MAT***a** *his1* and DC17 *MAT*α *his1*) and by bioassay for production of α-factor biological activity (15). Genomic DNAs from all of the stable (*ho*) α spores obtained were screened as described below for the presence of an *Eco*RI fragment of the size characteristic of either *S. cerevisiae* or *S. oviformis*. Although only 12 stable α spores were analyzed from the 50 tetrads dissected, 5 contained the larger fragment of the *S. oviformis* parent and the remainder had the smaller fragment from the *S. cerevisiae* parent, in agreement with the 2:2 segregation pattern expected for a single nuclear gene. Media, growth conditions, and genetic manipulations were all as described by Sherman et al. (30).

Plasmid DNAs and gene probes. Initial experiments were performed with a probe derived from a plasmid (p69A) containing the putative α-factor structural gene, generously provided by J. Kurjan and I. Herskowitz. This plasmid was isolated (18) from a library (24) of DNA segments from *S. cerevisiae* AB320 in the vector YEp13 (4). All of the preliminary studies were repeated and all subsequent experiments were performed with a probe derived from a plasmid (pAB101) isolated from a library (6) of DNA segments from another *S. cerevisiae* strain (DBY939) in the vector YEp24 (3), originally constructed by Chevallier et al. (8). pAB101 was identified by screening the library of individual *Escherichia coli* transformants by colony hybridization (12), using a 20-base-long synthetic deoxyribonucleotide (5'-TTAGTACATTGGTTGGCCGG-3'; kindly prepared by James Merryweather, Chiron Corporation, Emeryville, Calif.) This oligonucleotide is complementary to the DNA sequence obtained by Kurjan and Herskowitz (18) for the C-terminal end of the region apparently coding for prepro-α-factor. The nucleotide sequence corresponding to the entire coding region of our isolate was determined by the dideoxynucleoside triphosphate chain-termination method (28) after subcloning appropriate restriction fragments into bacteriophage M13 vectors (13; A. Brake and R. Najarian, unpublished results) and differs from that obtained by Kurjan and Herskowitz (18) in only one base. Our sequence contains a T at nucleotide 125 (instead of the C reported previously), and hence residue 42 of prepro-α-factor is presumably leucine (and not serine). This difference could reflect a trivial sequence polymorphism between the prepro-α-factor genes obtained from the two different *S. cerevisiae* strains due to a silent mutation.

The prepro-α-factor gene in pAB101 is contained in a 9-kilobase (kb) insert. To prepare the probe used for Southern blot and other hybridization analyses, pAB101 was cleaved to completion with *Eco*RI, and the resulting fragments were separated by agarose gel electrophoresis. The 1.8-kb *Eco*RI fragment containing the intact α-factor structural gene was extracted from the gel by standard methods (21) and ligated into the single *Eco*RI site of pAB11. pAB11 is a derivative of pBR322 lacking the region between the single *Hin*dIII and *Sal*I sites. This vector was constructed by digesting pBR322 to completion with *Hin*dIII and *Sal*I followed by limited digestion with *Bal*31 nuclease, repair of the ends so created with the Klenow fragment of *E. coli* DNA polymerase I, and blunt-end ligation with T4 DNA ligase to reform closed covalent circles.

DNA and RNA isolation. DNA was isolated from cultures (50 ml) of yeast cells grown to saturation in

TABLE 1. Yeast strains

Strain	Species	Genotype	Origin
X2180-1B	*S. cerevisiae*	*MAT*α *SUC2 mal gal2 CUP1*	YGSC[a]
121	*S. cerevisiae*	*MAT*α *ste5-3 leu2 trp1 ade2*	L. Hartwell
XT1172-S245C	*S. cerevisiae*	*MAT*α *ade6 his6 met1 leu1 trp5 gal2 can1 rme1*	V. MacKay
DBY939	*S. cerevisiae*	*MAT*α *ade2-101 suc2-215*(Am)	M. Carlson
DCO22	*S. carlsbergensis*	*MAT***a** *ade1 his4 leu2* [cir^0]	CSH[b]
2106-13-1	*S. carlsbergensis*	*MAT*α *ade1 nib*$^-$ [cir^0]	C. Holm
Y55	*S. carlsbergensis*	*MAT***a**/*MAT*α *HO/HO*	J. Haber
278	*S. diastaticus*	*MAT***a**/*MAT*α *HO/HO*	CSH
4346-2B$_{4-2}$	*S. douglasii*	*MAT*α *HML*α *HMR*α *HO ura3* (*ade*$^-$)	D. Hawthorne
Y4288-26	*S. kluyveri*	*MAT*α	C. Ballou
274	*S. norbensis*	*MAT***a**/*MAT*α *HO/HO*	CSH
273	*S. norbensis*	*MAT*α *HML*α *HMR*α *HO*	CSH
279	*S. oviformis*	*MAT***a** *HML***a** *HMR***a** *HO*	CSH
277	*S. oviformis*	*MAT***a** *HML***a** *HMR***a** *HO ade1 his4 leu2 thr4 gal1*	L. Blair
AB723	Hybrid[c]	*MAT*α *his6* (*his4*) *thr4*	This work

[a] YGSC, Yeast Genetic Stock Center, Donner Laboratory, University of California, Berkeley, Calif.
[b] CSH, Yeast Group, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
[c] Constructed by crossing an *S. oviformis* strain and an *S. cerevisiae* strain, as described in the text.

YPD. Cells were suspended in 3.5 ml of 0.9 M sorbitol–0.1 M EDTA–14 mM 2-mercaptoethanol (pH 7.5) and converted to spheroplasts by treatment with 100 μg of Zymolyase 60,000 (Kirin Brewery) per ml for 60 min at 37°C. Spheroplasts were collected by brief low-speed centrifugation, suspended in 3.5 ml of 10 mM Tris-hydrochloride–1 mM EDTA (pH 7.5) (TE), and lysed by addition of 1/10 volume of 0.5 M EDTA (pH 8.5) and 1/20 volume of 2 M Tris-hydrochloride (pH 7.5) followed by 0.5 ml of 10% sodium dodecyl sulfate. To complete lysis and DNA solubilization, we incubated the mixtures for 30 min at 68°C. To remove detergent and coprecipitate protein, we added 5 M potassium acetate (0.8 ml), and the suspension was chilled on ice for 1 h. After removal of the insoluble material by centrifugation at 15,000 × *g* for 15 min, nucleic acids were precipitated by the addition of 2 volumes of ethanol, collected by centrifugation, and suspended in 3 ml of TE. The solution was digested with 100 μg of RNase A (Worthington) per ml at 68°C for 15 min and then extracted with 3 ml of chloroform-isoamyl alcohol (24:1). The aqueous phase was removed to a fresh tube, and DNA was precipitated by addition of 3 M sodium acetate (0.3 ml) and isopropanol (2 ml). The DNA pellets were rinsed with 70% ethanol, dried, and redissolved in 0.5 ml of TE.

RNA was isolated from yeast cultures grown in YPD to mid-exponential phase (about 2×10^7 per ml). Total RNA and polyadenylated [poly(A)$^+$] RNA were prepared by only minor modifications of the procedures of Sripati and Warner (33).

Filter hybridizations to DNA and RNA. DNA was digested by restriction endonucleases as recommended by the suppliers (New England BioLabs or Bethesda Research Laboratories). Fragments were separated electrophoretically in agarose gels (0.8 to 2%) (23) and transferred to nitrocellulose filters (Sartorius) according to the method of Southern (31). Glyoxal-treated RNA was subjected to electrophoresis and transferred to nitrocellulose according to the procedure of Thomas (37).

Hybridizations were conducted under the conditions described by Wahl et al. (39), using denatured double-stranded DNA probes that were labeled with [α-^{32}P]deoxyribonucleoside triphosphates (Amersham) by nick translation (27) to specific activities of 5×10^7 to 3×10^8 cpm/μg, or, under conditions of reduced stringency (9), using the complementary oligonucleotide that was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase.

Molecular weight markers were a mixture of three separate restriction endonuclease digests (*Eco*RI, *Taq*I, and *Rsa*I) of pBR322 (35).

In vitro translation of yeast mRNA. In vitro translation reactions were performed with a nuclease-treated wheat germ extract (Bethesda Research Laboratories). The system was programmed either with total poly(A)$^+$ RNA (10 μg) or with poly(A)$^+$ RNA hybrid-selected with nitrocellulose filter squares (5 mm^2) on which were immobilized either pAB11 or the same plasmid containing the 1.8-kb insert with the α-factor structural gene, following the procedures of Paterson and Roberts (25). Reaction mixtures (final volume, 30 μl) contained 20 μCi of [^{35}S]methionine. The concentrations of the other components of the reaction mixture were as specified by the manufacturer. Incubations were conducted for 75 min at 25°C. Identical results were obtained with a nuclease-treated rabbit reticulocyte system (New England Nuclear), following the recommendations of the supplier.

Immunoprecipitations and polyacrylamide gel electrophoresis. In vitro translation reaction mixtures primed with total poly(A)$^+$ RNA were dried in a centrifugal concentrator (Savant Speed-Vac) and redissolved in 50 μl of immunoprecipitation buffer (IPB): 1% Triton X-100–0.5% sodium deoxycholate–0.1% sodium dodecyl sulfate in phosphate-buffered saline. A sample (50 μl) of a 10% (vol/vol) suspension of fixed *Staphylococcus aureus* Cowan I cells (IgG-Sorb, New England Enzyme Center) in IPB containing 5 mg of ovalbumin per ml was added. The mixture was incubated on ice for 30 min. The cells and the nonspecifically adsorbed material were collected by centrifugation at 12,000 × *g* for 10 min. The supernatant fluid was removed to a fresh tube and either anti-α-factor serum (5 μl) or nonimmune serum (5 μl) was added. Complex formation was allowed to proceed on ice for 60 min, after which immune complexes were adsorbed according the method described by Kessler (16). An additional volume (50 μl) of the 10% (vol/vol) suspension of *S. aureus* cells in IPB containing 5 mg of ovalbumin per ml was added, and the mixture was incubated on ice for 30 min. Cells and the adsorbed immune complexes were collected by centrifugation and washed twice with 0.5 ml of IPB containing 0.5 mg of ovalbumin per ml and 0.5% NaCl and twice with 0.5 ml of IPB containing 0.5 mg of ovalbumin per ml. The adsorbed immune complexes were collected by centrifugation, suspended in sample buffer (10 M urea, 2% sodium dodecyl sulfate, 30 mM dithiothreitol, 50 mM Tris-hydrochloride [pH 8.0]), and solubilized by boiling for 3 min. The boiled mixture was clarified by centrifugation at 12,000 × *g* for 5 min, and a sample containing an appropriate amount of radioactivity was removed for analysis by polyacrylamide gel electrophoresis, which was conducted in slabs of 15% gel according to the method of Laemmli (19). Either ^{14}C-labeled low-molecular-weight marker proteins (Bethesda Research Laboratories) or a set of cyanobacterial phycobiliproteins of known amino acid sequence (11) were used as the molecular weight standards. Typically, about 0.5 to 1.0% of the total radioactivity incorporated into acid-precipitable material by the wheat germ system was immunoprecipitable with anti-α-factor antibody and somewhat less (0.3 to 0.5%) by the rabbit reticulocyte system.

In vitro translation reaction mixtures primed with the hybrid-selected poly(A)$^+$ RNAs were also dried in the centrifugal concentrator but were suspended directly in an appropriate volume of sample buffer, boiled, and clarified, and a sample was removed for analysis as described above.

Autoradiography. Autoradiography of nucleic acid blots was performed at −80°C, using Kodak XAR film and intensifying screens (20). Fluorography of polyacrylamide gels, after impregnation with salicylate (7), was conducted at −80°C with preflashed film (2).

RESULTS

α-Factor structural gene differs among *Saccharomyces* species. To determine whether the prepro-α-factor gene is a conserved DNA segment, we examined the structure of this region

from several different strains of *S. cerevisiae* and from other related *Saccharomyces* species by Southern blot hybridization analysis of restriction endonuclease digests of genomic DNA from these organisms. The hybridization probe used in this study was a unique 1.8-kb *Eco*RI fragment which contains the entire coding region for the putative prepro-α-factor gene of *S. cerevisiae* (18), as well as nearly 0.9 kb of the 5'-flanking sequence and about 0.4-kb of the 3'-flanking sequence of this gene (Fig. 1A).

When digested with *Eco*RI, DNA from all the closely related *Saccharomyces* yeasts examined yielded a single fragment which displayed strong hybridization to the probe at high stringency (Fig. 2). For every strain purported to be *S. cerevisiae* and for an *S. norbensis* strain, these *Eco*RI fragments were apparently identical in size to that of the *Eco*RI segment cloned from two different *S. cerevisiae* stocks (see above). It was noted, however, that several other *Saccharomyces* yeasts reproducibly yielded *Eco*RI pieces which showed slight differences in size from that of *S. cerevisiae*. Based on their mobility relative to markers of known molecular weight, *Saccharomyces carlsbergensis* and *Saccharomyces diastaticus* gave *Eco*RI fragments that were approximately 50 bp shorter than the corresponding fragment from *S. cerevisiae*; conversely, *S. oviformis* displayed an *Eco*RI fragment that was about 50 bp longer.

In a less closely related species, *Saccharomyces douglasii*, the probe recognized a 3-kb *Eco*RI fragment but hybridized only weakly at the stringency used. In an even more distantly related species, *Saccharomyces kluyveri*, no detectable hybridization by the probe was observed under these same conditions (results not shown).

Polymorphisms due to differences in the length of the prepro-α-factor coding region. To determine more precisely the physical basis for the observed polymorphisms, we digested genomic DNAs from the same spectrum of *Saccharomyces* yeasts with several additional restriction endonucleases, which would give different diagnostic patterns for this chromosomal region. From extensive mapping of the restriction endonuclease cleavage sites in this region of *S. cerevisiae* and confirmation by nucleotide sequence analysis of the cloned DNA, it is known that *Pst*I cleaves at the amino-terminal end of the prepro-α-factor coding sequence (immediately after amino acid 9) and *Sal*I cuts after the carboxyl-terminal end of the coding region (35 base pairs (bp) after the ochre stop codon). Thus, almost the entire prepro-α-factor coding region of *S. cerevisiae* is contained on the 506-bp fragment generated by double digestion with *Pst*I and *Sal*I (Fig. 1B). Therefore, genomic DNAs were digested with both *Pst*I and *Sal*I and hybridized to the same cloned *S. cerevisiae* *Eco*RI fragment. Again, based on their mobility relative to markers of known molecular weight, the corresponding *Pst*I-*Sal*I fragments from *S. carlsbergensis* and *S. diastaticus* were found to be about 60 bp smaller than that of *S. cerevisiae*, and the corresponding *Pst*I-*Sal*I segment from *S. oviformis* was found to be about 60 bp larger (Fig. 3). Thus, the polymorphisms observed in the larger *Eco*RI fragments were confined to the protein coding region or, in part, to a short stretch of 3'-noncoding sequence immediately adjacent to the gene.

To confirm that the observed size differences were restricted exclusively to the prepro-α-factor coding region and did not involve the 3'-flanking sequence, we performed a different double digestion. It was known also from restriction endonuclease mapping studies and from the DNA sequence that there are four closely spaced *Hin*dIII cleavage sites that all lie within the protein coding region of the *Eco*RI fragment from *S. cerevisiae* (Fig. 1). In fact, these *Hin*dIII sites occur in each of the four tandem repeats of the α-factor coding sequences of the putative precursor, near the junction of a spacer peptide and its trailing mature phero-

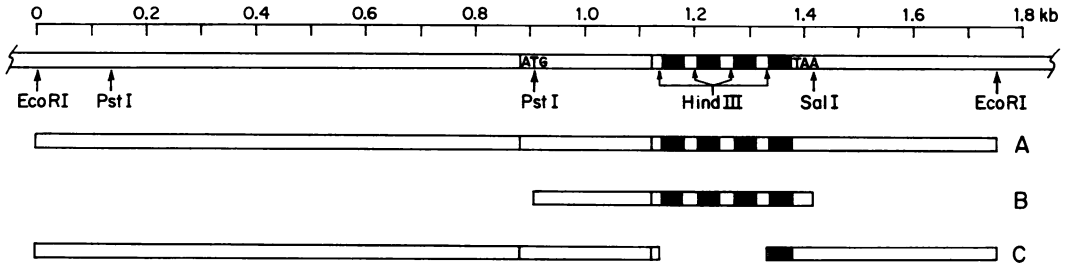

FIG. 1. Schematic representation of the chromosomal region containing the structural gene for prepro-α-factor in *S. cerevisiae*. Fragments of the indicated size are generated by digestion of genomic DNA with the following restriction endonucleases: *Eco*RI (A), *Pst*I + *Sal*I (B), and *Eco*RI + *Hin*dIII (C).

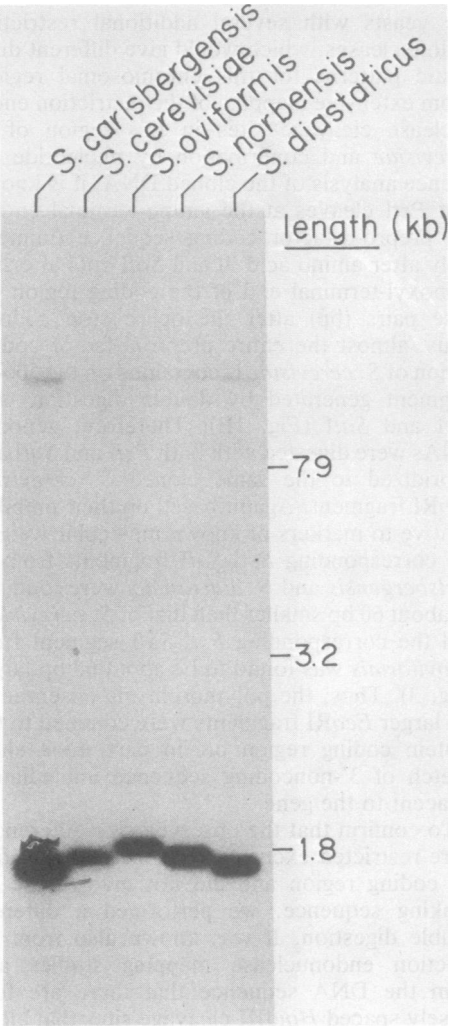

FIG. 2. Region containing the prepro-α-factor gene with species-specific length polymorphisms of limited size. Genomic DNAs purified from the indicated yeast species were digested to completion with *Eco*RI, subjected to electrophoresis in a 0.8% agarose gel at 40 V overnight, transferred to nitrocellulose, hybridized to a ^{32}P-labeled probe containing the 1.8-kb *Eco*RI fragment from *S. cerevisiae* (Fig. 1A) in a derivative of pBR322, and examined by autoradiography.

mone sequence. Hence, digestion with both *Eco*RI and *Hin*dIII (Fig. 1C) produces: (i) an approximately 1.2-kb fragment containing the 5′-noncoding region of the gene, the N-terminal half of prepro-α-factor, including one copy of the spacer peptide, but no α-factor information; (ii) an approximately 0.35-kb fragment containing the 3′-noncoding region of the gene and just the C-terminal-most copy of pheromone sequence; and (iii) three small, essentially identical 63-bp fragments corresponding to the three internal copies of spacer peptide/pheromone coding segments.

After digestion of the genomic DNAs with both *Eco*RI and *Hin*dIII and hybridization with the same probe used in the previous studies, it was found that the two fragments corresponding to the 5′- and 3′-flanking regions were identical in size in all the *Saccharomyces* species examined (Fig. 4). (The 63-bp fragments were not observed because they migrated off the agarose gel under the electrophoresis conditions used.) Hence, this result demonstrated that the size differences seen in both the *Eco*RI (Fig. 2) and *Pst*I-*Sal*I (Fig. 3) digests must be located within the tandemly arranged spacer peptide/pheromone coding regions of the precursor. Furthermore, within the resolution of this technique, the observed size differences of ±60 bp between the other *Saccharomyces* species and *S. cerevisiae* correspond exactly to the size (63 bp) of a single

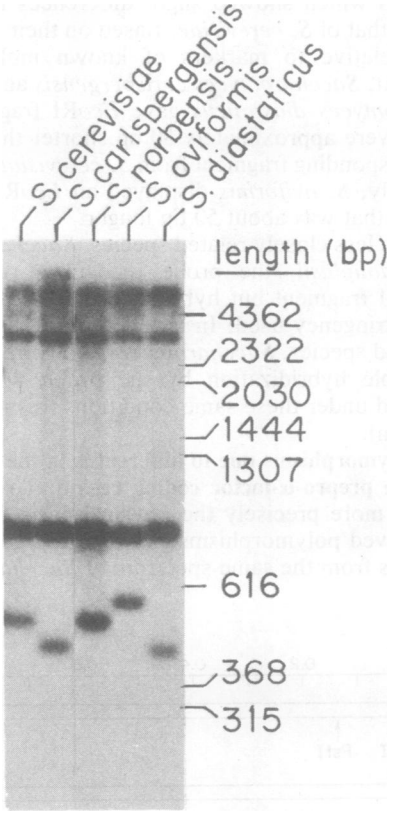

FIG. 3. Prepro-α-factor coding regions containing the species-specific length polymorphisms. Genomic DNAs of the indicated yeasts were digested to completion with both *Pst*I and *Sal*I, subjected to electrophoresis in a 2% agarose gel at 50 V for 12 h, transferred to nitrocellulose, hybridized to the probe described in the legend to Fig. 2, and examined by autoradiography.

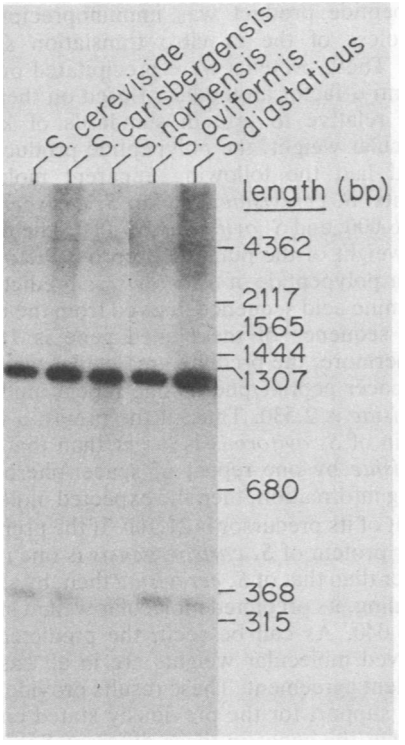

FIG. 4. Prepro-α-factor gene 5'- and 3'-flanking regions without length polymorphisms. Genomic DNAs of the indicated species were digested to completion with both *Eco*RI and *Hin*dIII, subjected to electrophoresis in a 2% agarose gel at 120 V for 5 h, transferred to nitrocellulose, hybridized to the probe described in the legend to Fig. 2, and examined by autoradiography.

repeat of the spacer peptide/pheromone coding information. Hence, the simplest interpretation of these results is that the lengths of the prepro-α-factor genes of the various yeast species differ only in the number of tandem α-factor coding sequences carried. Thus, *S. cerevisiae* has four pheromone coding units as presumably does *S. norbensis*; but *Saccharomyces oviformis* probably has five α-factor coding units, and *S. carlsbergensis* and *S. diastaticus* most likely have only three.

Correlation of the sizes of the RNA transcripts with the apparent number of repeats of pheromone sequence within the prepro-α-factor genes. If the genomic DNA sequences characterized by the Southern blot analysis are functional (expressed) genes that differ only in the number of α-factor repeats specified, then the size differences observed in the putative prepro-α-factor coding regions should also be reflected in the mRNA molecules transcribed from these genes. To determine whether this was indeed the case, we examined poly(A)$^+$ RNAs isolated from the various *Saccharomyces* yeasts by Northern blot hybridization analysis, using the cloned *S. cerevisiae* DNA as the probe.

Production of α-factor is an α-cell-specific function (34). Because synthesis of α-factor only occurs in haploid cells of the α mating type, it was anticipated that to perform an analysis of prepro-α-factor mRNA, *MAT*α haploids carrying the three different polymorphic forms of the gene would be required. Poly(A)$^+$ mRNAs were prepared from the same heterothallic (stable) *MAT*α haploids of *S. cerevisiae* and *S. carlsbergensis* that were used for the Southern blot analysis of genomic DNA. The *S. oviformis* strain previously analyzed was, however, a homothallic a cell (14). Therefore, a stable *MAT*α haploid containing the prepro-α-factor gene of *S. oviformis* was derived by genetic crosses and confirmed by subsequent analysis as described above.

The lengths of the prepro-α-factor mRNAs produced by these three yeast strains apparently differed from each other by the same number of nucleotides as was observed for their cognate genes (Fig. 5). Specifically, the prepro-α-factor mRNA of *S. carlsbergensis* was about 65 nucleotides shorter than that from *S. cerevisiae* (825 nucleotides), and the prepro-α-factor mRNA of *S. oviformis* was about 65 nucleotides longer. These same transcripts were the only species detected when the blots were reprobed at low stringency with the synthetic 20 base-long oligonucleotide (complementary to just the pheromone coding sequence) used to clone the *S. cerevisiae* prepro-α-factor gene (data not shown).

These results demonstrate unequivocally that the putative prepro-α-factor genes are, in fact, transcribed in *MAT*α haploids. In addition, the observation that the RNA transcripts directly reflect the size differences found for the genes themselves provides further support for the conclusion that the genes differ in the size of the prepro-α-factor precursor proteins that they encode.

Prepro-α-factor proteins of the sizes predicted by the lengths of the genes. To establish that the mRNA species detected were truly functional in that they encoded prepro-α-factor precursor polypeptides, we performed in vitro translation of the RNA. The products of the in vitro translations were analyzed by two complementary methods: immunoprecipitation of the proteins directed by total poly(A)$^+$ RNA, using anti-*S. cerevisiae* α-factor antibodies, and hybridization-selection of prepro-α-factor mRNA before its translation, using the cloned putative *S. cerevisiae* prepro-α-factor gene.

First, total poly(A)$^+$ RNA preparations from

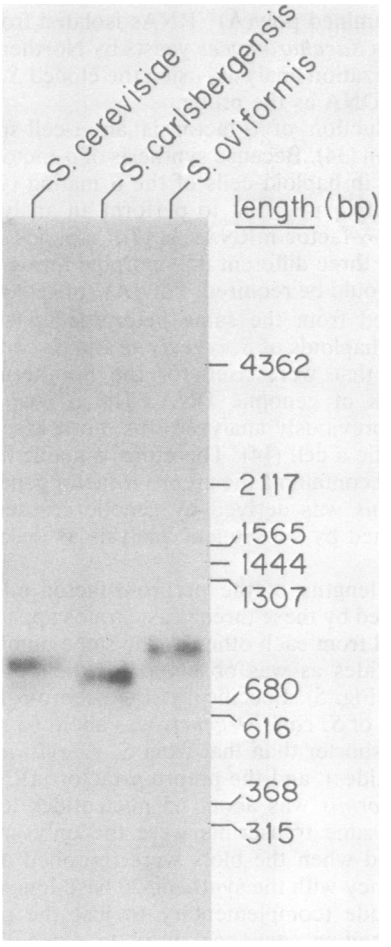

FIG. 5. Complementary poly(A)$^+$ RNAs reflect the sizes of the prepro-α-factor genes. Poly(A)$^+$ RNA was prepared from *MAT*α cells containing prepro-α-factor genes characteristic of the three different yeast species indicated, as described in the text. A sample (5 μg) of each preparation was denatured by treatment with glyoxal, subjected to electrophoresis in a 2% agarose gel at 50 V for 12 h, transferred to nitrocellulose, hybridized to the probe described in the legend to Fig. 2, and examined by autoradiography.

the same three *Saccharomyces* yeasts examined by Northern blot analysis were translated in both the wheat germ and the rabbit reticulocyte in vitro protein synthesizing systems, using [^{35}S]methionine as the label. The in vitro translation products were immunoprecipitated either with rabbit antiserum directed against mature α-factor or with nonimmune rabbit serum. The immunoprecipitates were then solubilized and subjected to electrophoresis in slabs of polyacrylamide gel containing sodium dodecyl sulfate (Fig. 6). From all three species, only a single polypeptide product was immunoprecipitated, regardless of the in vitro translation system used. These proteins were precipitated only by the anti-α-factor antibodies. Based on their mobility relative to protein standards of known molecular weight, the polypeptide products detected had the following apparent molecular weights: *S. cerevisiae*, 18,500; *S. carlsbergenesis*, 16,000; and *S. oviformis*, 21,000. The molecular weight of the putative prepro-α-factor precursor polypeptide of *S. cerevisiae* predicted by the amino acid sequence derived from the nucleotide sequence of the cloned gene is 18,600. Furthermore, the predicted molecular weight of the spacer peptide/pheromone repeat unit of *S. cerevisiae* is 2,530. Thus, if the prepro-α-factor protein of *S. oviformis* is larger than that of *S. cerevisiae* by one repeat of spacer/pheromone coding information, then the expected molecular weight of its precursor is 21,100. If the prepro-α-factor protein of *S. carlsbergensis* is one repeat shorter than that of *S. cerevisiae* then, by similar reasoning, its predicted molecular weight should be 16,040. As can be seen, the predicted and observed molecular weights are in all cases in excellent agreement. These results provide additional support for the previously stated conclusion that the genes in these different *Saccharomyces* species encode functional prepro-α-factor

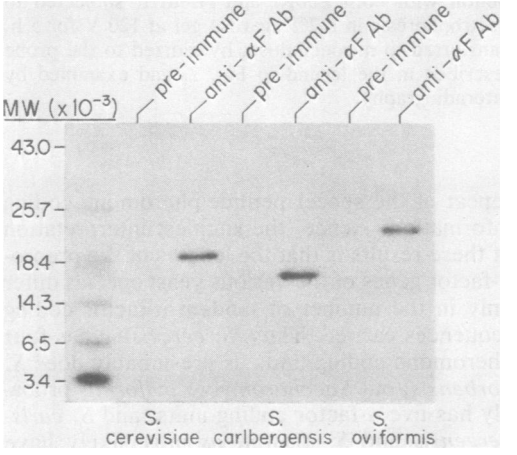

FIG. 6. Analysis of prepro-α-factor proteins by immunoprecipitation of in vitro translation products. In vitro translation reactions were programmed with total poly(A)$^+$ RNAs prepared from *MAT*α cells of the three different yeast species indicated, as described in the text. ^{35}S-labeled proteins antigenically related to α-factor were immunoprecipitated, subjected to electrophoresis in a slab of polyacrylamide, and examined by fluorography, as described in the text. Approximately 5,000 cpm of the material immunoprecipitated with anti-α-factor serum (anti-αF′ Ab) and the total material from the preimmune serum controls (about 2,500 cpm) were loaded in the gel wells.

precursors that essentially differ only in the number of pheromone repeat units present in the molecule.

For assurance that the observed polypeptides were products solely derived from the mRNA species detected in the Northern blot hybridizations and were not encoded by other molecules in the total poly(A)$^+$ RNA preparations, the method of hybridization selection was used. The 1.8-kb *Eco*RI fragment containing the *S. cerevisiae* prepro-α-factor gene was inserted into the single *Eco*RI site of a pBR322 derivative. This plasmid and control vector DNA were immobilized on separate nitrocellulose filters and used to select complementary mRNA molecules from poly(A)$^+$ RNA preparations from the three different *Saccharomyces* species. The RNA which hybridized to the filters was eluted and used to direct either the rabbit reticulocyte or the wheat germ protein synthesizing systems. The total ^{35}S-labeled protein products of the in vitro translation were then analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 7).

When programmed with control RNAs (hybrid selected with the vector alone), no new protein species aside from those specified by the endogenous RNA were detectable, as expected. In contrast, when programmed with the complementary RNAs hybrid selected with the plasmid containing the *S. cerevisiae* prepro-α-factor gene, only one new polypeptide appeared in each of the three different reactions. Moreover, each of these new polypeptides was identical in molecular weight to that found by immunoprecipitation. These results confirmed that the prepro-α-factor polypeptides observed by immunoprecipitation were the specific products of the transcripts of the prepro-α-factor genes detected in the Southern and Northern analyses.

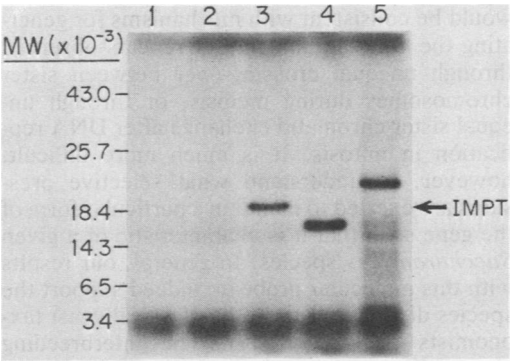

FIG. 7. Analysis of prepro-α-factor proteins by hybridization-selection of poly(A)$^+$ mRNAs and in vitro translation. Poly(A)$^+$ mRNAs complementary to the prepro-α-factor gene of *S. cerevisiae* were selected from the total poly(A)$^+$ RNA populations of *MAT*α cells of the indicated yeast species by hybridization to the cloned DNA in a pBR322 derivative, and controls were obtained by hybridizing the same RNAs to the vector alone, as described in the text. The hybrid-selected RNAs were used to program in vitro translation reactions and the total products were examined (without prior immunoprecipitation) by polyacrylamide gel electrophoresis and fluorography. Lane 1, no exogenous RNA added (12,300 cpm); lane 2, *S. cerevisiae* RNA selected with pAB11 (16,000 cpm); lane 3, *S. cerevisiae* RNA selected with pAB11 containing the α-factor gene (29,300 cpm); lane 4, *S. carlsbergensis* RNA selected with pAB11 containing the α-factor gene (60,000 cpm); lane 5, *S. oviformis* RNA selected with pAB11 containing the α-factor gene (32,000 cpm). Results identical to the control shown in lane 2 were obtained if either *S. carlsbergenesis* or *S. oviformis* RNAs was hybrid selected with just pAB11 alone. A sample of the material shown in lane 3 also was subjected to immunoprecipitation before electrophoresis and only a single band with a mobility identical to that of the major product was found (arrow, IMPT).

DISCUSSION

The results presented here indicate that three *Saccharomyces* yeasts possess at least one expressed and functional structural gene for the α pheromone precursor. By comparing restriction endonuclease cleavage site maps of this α-factor gene from several different yeast species, we found that the structure of the gene is highly conserved, but it is polymorphic with respect to the number of spacer peptide/mature pheromone coding units that are present. These conclusions were corroborated by comparing the sizes of the mRNAs encoding prepro-α-factor from these yeast species and by analyzing the precursor proteins synthesized in vitro in translation systems primed with these mRNAs.

At least four different stocks of *S. cerevisiae*, representing lineages separately maintained and propagated in Berkeley (X2180-1B), Boston (DBY939), Chicago (AB320), and Seattle (121), all possess prepro-α-factor genes that contain four tandemly arrayed spacer peptide/pheromone coding regions. This same situation occurs in the two different *S. norbensis* strains examined (273 and 274). In contrast, in three independently maintained *S. carlsbergensis* strains (DC022, 2106-13-1, and Y55) and in an *S. diastaticus* strain (278), the α-factor gene is smaller because it appears to contain only three repeats of the spacer peptide/pheromone coding information. In an *S. oviformis* strain (279), however, the gene is larger because it apparently has five repeats.

The fact that these genes contain multiple repeats of highly homologous short sequences

would be consistent with mechanisms for generating the variable numbers of repeats observed through unequal crossing-over between sister chromosomes during meiosis, or through unequal sister chromatid exchange after DNA replication in mitosis. It is much more difficult, however, to understand what selective pressures are exerted to maintain a particular form of the gene such that it is characteristic of a given *Saccharomyces* species. In general, our results with this molecular probe do indeed support the species distinctions made by classical yeast taxonomists previously (17). The interbreeding yeasts, *S. cerevisiae*, *S. norbensis*, *S. carlsbergensis*, *S. diastaticus*, and *S. oviformis*, have very similar gene arrangements, whereas the poorly interfertile species *S. douglasii* has an α pheromone gene that appears only somewhat related by the criteria of showing only weak hybridization to the *S. cerevisiae* gene and an apparent polymorphism for an *Eco*RI cleavage site. *S. kluveryi* will not conjugate at all with *S. cerevisiae* (5, 22) and correspondingly its α-factor gene appeared to be essentially unrelated at the nucleic acid level. In fact, it has been recently reported that even the mature α pheromone of *S. kluyveri* differs in amino acid sequence from that of *S. cerevisiae* in at least four positions (29).

We found in this work that in all the related yeasts examined, in addition to the structural gene itself, a very large *Eco*RI fragment (about 15 kb) was also recognized, but only very weakly, by our gene probe. A similar observation was made and noted by Kurjan and Herskowitz (18) for the strain of *S. cerevisiae* that they studied. Remarkably, this large *Eco*RI fragment displayed as good or better hybridization to the synthetic oligonucleotide complementary to the coding sequence of the mature pheromone which we used to clone the *S. cerevisiae* gene (A. Brake, unpublished results). This finding confirms that this chromosomal region at least contains pheromone coding information. By using either the gene probe or the oligonucleotide probe, however, only transcripts corresponding to the size expected for the structural gene were detected upon analysis of poly(A)$^+$ mRNAs from the three related yeast species examined in detail. Hence, the very large *Eco*RI fragment may harbor some sort of inactive (unexpressed) "pseudogene" or "processed gene." Alternatively, this region may be expressed, but only at a very low level, or may be expressed only under special conditions (for example, induction by **a**-factor), or, less likely, may produce a transcript identical in size to that from the structural gene. Yet another possibility is that this region contains sequences for maintaining the integrity of the coding sequences within the expressed structural gene by some sort of inter- or intrachromosomal gene conversion process. Clearly, the precise structure of the apparent pheromone coding region in the very large *Eco*RI fragment is of interest, and we have cloned this particular segment to permit its detailed characterization (A. Brake, unpublished results).

The lengths of the complementary RNAs obtained from *MAT*α cells of three different yeast species reflected exactly the lengths of their cognate genes. No transcript complementary to either the cloned *S. cerevisiae* α-factor gene or the synthetic oligonucleotide probe could be detected in any *MAT***a** haploid or in any *MAT***a**/*MAT*α diploid tested (J. Thorner and A. Brake, unpublished results), confirming that expression of the α-factor gene is indeed an α-cell-specific function. The transcript from *S. cerevisiae* *MAT*α cells that we detected by gel electrophoresis and blotting was significantly larger (825 nucleotides) than the size (500 nucleotides) reported by Kurjan and Herskowitz (18) on the basis of preliminary R-loop analysis. Our current understanding of the mechanism of yeast transcription can readily explain a transcript of the size we observed. The α-factor structural gene of *S. cerevisiae* has a potential recognition site for RNA polymerase II (TATATAA) at a position −128 bp upstream from the presumptive initiator Met codon. Other cloned yeast genes contain a very similar or identical sequence in essentially the same position, for example: *SUC2*, TATAAATA, −133 (36); *ADH1*, TATAAATA, −128 (1); and *CYC1*, TATATAAAA, −124 (10). The position of the start of transcription is, however, quite variable among different yeast genes, and many individual yeast genes appear to have multiple transcriptional starts. Nevertheless, strong transcriptional starts almost invariably fall in the range from −70 to −30 bp proximal to the initiating ATG (e.g., *SUC2*, −40; *ADH1*, −37, −27; *CYC1*, −61, −46, −34). Presumably the start of transcription of the α-factor gene occurs in this same region, and, if this is in fact the case, the initiating Met of prepro-α-factor is indeed the first AUG a ribosome would encounter in the mRNA. On the basis of a compilation of the DNA sequences of the 3′-nontranslated regions flanking 15 different cloned yeast genes, Zaret and Sherman (40) have proposed a consensus arrangement for the sequences specifying termination of transcription by yeast RNA polymerase II: translational stop codon/spacer ($n \leq 140$)/T-rich region/spacer ($n \leq 5$)/TAG (or TAA)/spacer ($n \leq 14$)/TAGT (or TATGT)/AT-rich region/TTT. Addition of poly(A) tails generally occurs within 10 to 20 bp after the short terminal T tract. The 3′-flanking region of the α-factor

gene contains several very similar sets of sequences. One begins at +84 bp after the ochre stop codon: T-rich region/spacer (n = 5)/TAA/spacer (n = 14)/CATGT/AT-rich region/TTT. Assuming that these considerations provide reasonable estimates for the lengths of the 5'- and 3'-nontranslated regions, and keeping in mind that the prepro-α-factor coding region itself is 498 nucleotides and the fact that the average length of the poly(A) tract added to yeast mRNAs is about 60 bases or so (26), the predicted length for the transcript of the *S. cerevisiae* α-factor gene is about 800 nucleotides, which is in good agreement with the size observed experimentally. As revealed clearly by longer exposures of the Northern blots, a minor amount of a somewhat shorter transcript was also observed in all three yeast species (Fig. 5). This second species could reflect an alternate weak transcriptional start site (10) or could represent an alternate weak transcriptional termination site (1). To distinguish between these two possibilities, detailed mapping of the 5' and 3' ends of the mRNA is required and is in progress.

The apparent molecular weights of the proteins encoded by the transcripts of the three yeast species studied also corresponded exactly to those predicted from the sizes of their cognate genes, assuming that the genes differed only in the number of spacer peptide/pheromone repeat units within the prepro-α-factor coding region. In particular, the molecular weight of the in vitro translation product of the *S. cerevisiae* mRNA agreed extremely well with the size of the protein predicted for the prepro-α-factor precursor from the nucleotide sequence obtained by Kurjan and Herskowitz (18) and by ourselves (see above). That the structure of the in vitro translation product mirrors that predicted for prepro-α-factor is also suggested by an additional observation. The nucleotide sequence indicates that the translational stop codon immediately follows the C-terminal amino acid of the last copy of pheromone coding information, and hence the C-terminus of the last pheromone repeat should be in its mature form even in the precursor. The fact that the protein made in vitro was efficiently immunoprecipitated by antibodies directed against the C-terminus of the mature pheromone (15; Y. Jones-Brown, J. Thorner, A. Brake, and E. Ciejek, submitted for publication) indicates that the last pheromone repeat in the precursor does indeed have a mature C-terminus. The immunoreactivity of the in vitro translation product indicates that our antibodies should be a useful tool for determining whether this primary translation product can be detected transiently in yeast cells in vivo and, if so, for tracing the fate of this molecule during the maturation and secretion of α-factor. In this regard, based on the fraction of the total radioactivity incorporated into protein in vitro that was immunoprecipitable with anti-α-factor antibodies (0.5 to 1%), the transcript of the prepro-α-factor gene is a reasonably abundant mRNA. This result suggests further that the α-factor gene has a relatively strong promoter, at least in α cells.

Although two of the *S. cerevisiae MAT*α strains examined in this work (X2180-1B and XT1172-S245C) appear to contain α-factor genes of identical size and restriction pattern, these strains seem to release significantly different amounts of mature α-factor, as judged both by a sensitive bioassay and by a radioimmunoassay (15). Hence, parameters other than simply the number of tandem repeats of pheromone coding units within the gene influence the efficiency of α-factor production by α cells. These parameters may include subtle changes within the structural gene itself, which would be revealed only by obtaining the entire nucleotide sequence of individual α-factor genes from several different *S. cerevisiae* strains, or may involve other aspects of the biosynthesis, processing, and transport of the precursor protein. These alternatives are currently under investigation. Because all three types of prepro-α-factor genes studied permit *MAT*α cells to secrete α-factor, the number of spacer peptide/pheromone repeat units within the precursor is obviously not an essential determinant for proper processing of this molecule. It is likely, therefore, that the extremely hydrophilic nature of the spacer peptide regions, —LysArgGluAlaAsp(or Glu)AlaGluAla—, ensures that these portions of the precursor will be exposed on the outer surface, no matter how the precursor folds. These sites will thus be freely accessible to the appropriate processing enzymes.

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