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Multiple Genes Coding for Precursors of Rhodotorucine *A*, a Farnesyl Peptide Mating Pheromone of the Basidiomycetous Yeast *Rhodosporidium toruloides*

RINJI AKADA,[1]* KENJIRO MINOMI,[2] JINGO KAI,[2] ICHIRO YAMASHITA,[1] TOKICHI MIYAKAWA,[2] AND SAKUZO FUKUI[2]†

Center for Gene Science,[1] and Department of Fermentation Technology, Faculty of Engineering,[2] Hiroshima University, Saijo, Higashi-Hiroshima 724, Japan

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Haploid cells of mating type *A* of the basidiomycetous yeast *Rhodosporidium toruloides* secrete a mating pheromone, rhodotorucine *A*, which is an undecapeptide containing *S*-farnesyl cysteine at its carboxy terminus. To analyze the processing and secretion pathway of rhodotorucine *A*, we isolated both genomic and complementary DNAs encoding the peptide moiety. We identified three distinct genes, *RHA1*, *RHA2*, and *RHA3*, encoding four, five, and three copies of the pheromone peptide, respectively. Complementary DNA clones were classified into two types. One type was homologous to *RHA1*, and the other type was homologous to *RHA2*. Transcription start sites were identified by primer extension and S1 nuclease protection, from which the site of the initiator methionine was verified. A primary precursor of rhodotorucine *A* was detected as a 7-kilodalton protein by immunoprecipitation of in vitro translation products. On the basis of these results, we propose similar three-precursor structures of rhodotorucine *A*, each containing the amino-terminal peptide sequence Met-Val-Ala. The precursors contain three, four, or five tandem repeats of the pheromone peptide, each separated by a spacer peptide, Thr-Val-Ser(Ala)-Lys, and each precursor has the carboxy-terminal sequence Thr-Val-Ala. This structure suggests that primary precursors of rhodotorucine *A* do not contain canonical signal sequences.

Haploids of the basidiomycetous yeast *Rhodosporidium toruloides* secrete diffusible mating pheromones, rhodotorucine *A* and *a*, which elicit mating-tube formation in opposite mating-type cells (1). Rhodotorucine *A* has been purified and has been found to be an *S*-polyisoprenyl (*S*-farnesyl) undecapeptide with the following structure: H-Tyr-Pro-Glu-Ile-Ser-Trp-Thr-Arg-Asn-Gly-Cys(*S*-farnesyl)-OH (26). This novel lipopeptide is common to all mating pheromones of the basidiomycetous yeasts so far characterized (21, 41, 42).

In many higher eucaryotes, small peptide hormones and neuropeptides are processed proteolytically from larger precursor molecules (14). This processing of precursor polypeptides to produce biologically active peptides occurs also in unicellular eucaryotes. In the ascomycetous yeast *Saccharomyces cerevisiae*, a diffusible mating pheromone, α-factor, is produced by the processing of a larger precursor molecule, prepro-α-factor (29, 48). Prepro-α-factor contains a signal (pre) sequence of 19 hydrophobic amino acids at the amino terminus, followed by an additional 60-amino-acid sequence named the pro region. The carboxy-terminal half of prepro-α-factor contains four tandem repeats of the mature α-factor peptide, each 13 amino acids long, and each separated by spacer peptides with lengths of 6 to 8 amino acids (29). The steps involved in secretion of α-factor have been genetically and biochemically studied in detail, including the secretory pathway, glycosylation processes, and proteolytic maturation steps (23–25, 40). In addition, the precursor structure for **a**-factor, a mating pheromone secreted from **a**-type cells of *S. cerevisiae*, has been predicted, and its processing steps have been postulated (8, 18). Recently, the chemical structure of **a**-factor has been shown to include an *S*-farnesylated carboxy-terminal cysteine in common with that of rhodotorucine *A* (3). On the other hand, the biosynthetic and secretion mechanisms of other small secretory peptides in unicellular eucaryotes remain unknown.

Recently, lipid modifications of eucaryotic polypeptides, such as fatty acid acylation, have been recognized (45). For example, palmitic acid is added to $p21^{v\text{-}ras}$ (10) and the transferrin receptor (37), and myristic acid is attached to $p60^{v\text{-}src}$ (46). Although a definitive function for these lipid modifications has not been identified, it has been suggested that they might act as signals for transport to the plasma membrane and may be important in the interactions with cellular membranes. In the case of rhodotorucine *A*, the addition of a polyisoprenyl residue is involved in its maturation process. It is postulated that the polyisoprenyl nonpolar residue of rhodotorucine *A* interacts with the lipid bilayer of vesicles or of the plasma membrane and plays a role in its secretory pathway.

To investigate the biosynthesis and secretory pathway of rhodotorucine *A*, we isolated a gene (*RHA1*) encoding its peptide moiety by using synthetic oligonucleotides (2). The presence of another homologous sequence was found by genomic Southern blot analysis by using *RHA1* as a probe. Here, we report the cloning and sequencing of complementary and genomic DNAs encoding the peptide moiety of rhodotorucine *A*, from which the structure of the entire primary precursor structure is predicted. In addition, we prepared antibody directed against synthetic rhodotorucine *A* peptide and used it to detect the precursor molecule synthesized in vitro. These studies show that this novel lipopeptide pheromone is produced by processing from similar three precursors, differing only in the number of

* Corresponding author.
† Present address: Department of Biotechnology, Faculty of Technology, Fukuyama University, Fukuyama 729-02, Japan.

pheromone-spacer repeats, and that they do not have canonical signal sequences.

MATERIALS AND METHODS

Microorganisms. A haploid strain of *R. toruloides* IFO 0559-M919 (mating type *A*) was used (35). Yeast cells were grown aerobically in YPG medium containing 0.4% (wt/vol) yeast extract, 0.5% (wt/vol) polypeptone, 2% (wt/vol) glucose, 0.1% (wt/vol) KH_2PO_4, 0.05% (wt/vol) $MgSO_4 \cdot 7H_2O$ and 50 μg of chloramphenicol per ml.

Construction and screening of cDNA library. *A*-type cells of *R. toruloides* were cultured in 100 ml of YPG medium at 28°C to an optical density at 660 nm of 1.0. Total RNA was prepared from the cells by phenol-chloroform extraction (22) and poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography as recommended by the supplier (Collaborative Research, Inc.). The cDNA library of *R. toruloides* was constructed from poly(A)$^+$ RNA by using the λgt10 cDNA cloning system (Amersham Corp.) according to the method described by Huynh et al. (20). The cDNA library was amplified, and screening was performed by a standard plaque hybridization procedure. Filter replicas were made on nylon membranes (Pall Biodyne, Pall Ultrafine Filtration Corp.). The nylon membranes were prehybridized at 42°C for 4 h in a hybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 15 mM sodium citrate] [pH 7], 1× Denhardt solution (12), 50 mM sodium phosphate buffer [pH 6.5], 50% [vol/vol] formamide, 1 mg of cod sperm DNA per ml) and then hybridized overnight at 42°C in 4 ml of the hybridization solution containing labeled probes. The membranes were washed four times for 5 min each time in 2× SSC–0.1% sodium dodecyl sulfate (SDS) at room temperature and rewashed twice for 15 min each time in 0.1× SSC–0.1% SDS at 42°C. Then the membranes were dried and exposed to X-ray film with an intensifying screen at −70°C. Two probes were used for the screening (see Fig. 1): (i) a *Pvu*II-*Hin*dIII fragment from genomic clone pRA6 was inserted into pBR322 and labeled by nick translation (39), and (ii) a *Pvu*II-*Bgl*II fragment containing the 5′ upstream region of the pheromone-coding sequence was labeled with random hexamer oligonucleotide primers as described previously (16).

Cloning of additional *RHA* genes. Genomic DNA was digested with *Hin*dIII and was fractionated on 1% agarose gel. DNA fragments ranging from 8 to 10 kilobases (kb) in size were isolated with DEAE paper (15) and were cloned into pBR322, since Southern blot analysis showed that a 9-kb *Hin*dIII-digested fragment is present, in addition to a fragment (3.6 kb) derived from *RHA1* (data not shown). Colony hybridization was performed by a standard procedure by using cDNA clone 75, which was radiolabeled with random oligonucleotide primers, as a probe (16). The conditions for colony hybridization were the same as those for plaque hybridization described above.

DNA sequence determination. Genomic and complementary DNA fragments were subcloned into M13 vectors (mp10, mp11, mp18, and mp19) for sequencing. The DNA sequence was determined by the dideoxy-chain termination method (43).

Primer extension and S1 nuclease protection. An 18-mer oligonucleotide, 3′-AAGTTGTTGTACCAGCGG-5′, complementary to the 5′ upstream sequence of the pheromone coding region (see Fig. 4), was chemically synthesized. Primer extension was performed by the procedure of Domdey et al. (13). The 5′ end of the synthetic 18-mer oligonucleotide was labeled with [γ-^{32}P]ATP (5,000 Ci/mmol; ICN Radiochemicals Inc.) and T4 polynucleotide kinase (Toyobo Co.) and was purified with a NENSORB cartridge (DuPont, NEN Research Products) as recommended by the supplier. Hybridization was done in 45 μl of extension buffer (50 mM Tris hydrochloride [pH 8.3], 150 mM KCl, 0.5 mM EDTA [pH 7.5], 7 mM $MgCl_2$, 1 mM dithiothreitol) containing 5 ng of radiolabeled 18-mer oligonucleotide (1×10^6 cpm) and 20 μg poly(A)$^+$ RNA. The mixture was incubated at 65°C for 2 min and at room temperature for 5 min and was finally chilled on ice. Reverse transcription was started by adding 5 μl of 5 mM each of the four deoxyribonucleotide triphosphates and 20 U of RAV-2 reverse transcriptase (Takara Shuzo Co.), and the mixture was incubated at 42°C for 90 min. The RNA was then hydrolyzed by adding 12.5 μl of 0.5 M NaOH; the mixture was kept at 100°C for 3 min and then chilled on ice. The NaOH was neutralized by adding 12.5 μl of 0.5 M HCl and 12.5 μl of 1 M Tris hydrochloride, pH 7.5. The primer extension product was ethanol precipitated and fractionated on a polyacrylamide-urea sequencing gel. S1 nuclease protection was performed according to the procedure of Nasmyth (36). The ^{32}P-labeled fragment was prepared as follows. The 18-mer primer (5 ng) was hybridized with the single-stranded DNA of genomic *RHA1* cloned in M13. This procedure was followed by primer extension with Klenow fragment and [α-^{32}P]dCTP, and then double-stranded DNA synthesized was digested with *Hin*cII, which was located 260 base pair (bp) 5′ upstream of the priming site. The labeled single strand was isolated by using preparative polyacrylamide-urea tube gel electrophoresis. Hybridization was done at 42°C overnight in 25 μl of 50% (vol/vol) formamide–5× SSC–1× Denhardt solution minus bovine serum albumin containing 25 μg of poly(A)$^+$ RNA. A 300-μl portion of S1 nuclease buffer (4 mM $ZnSO_4$, 25 mM sodium acetate buffer [pH 4.6], 0.25 M NaCl) containing 200 U of S1 nuclease (Takara Shuzo Co.) was added to this mixture, and the mixture was incubated at 37°C for 45 min. The protected DNA fragment was ethanol precipitated and was fractionated on the sequencing gel. DNA sequence ladders were produced by hybridizing the 18-mer primer with *RHA1* DNA cloned in M13, followed by primer extension according to the dideoxy sequencing method, and they were used as size markers.

Construction of plasmid probes for the detection of *RHA* mRNAs. Plasmid probes were constructed by ligating pBR322 with a *Bgl*II-*Eco*RI fragment containing the 3′ noncoding regions of either cDNA clone 26 (span, 152 to 3′ end) or clone 75 (span, 197 to 3′ end), resulting in plasmids pJK-Sp1 and pJK-Sp2. An *Eco*RI-derived fragment of cDNA 14 (see Fig. 1) was inserted into the *Eco*RI sites of pJK-Sp1 and pJK-Sp2, resulting in pJK-Sp1-14 and pJK-Sp2-14, respectively. *Sal*I-*Pst*I fragments containing these insertions were used as probes.

Northern (RNA) blot hybridization. Poly(A)$^+$ RNA isolated from *R. toruloides A* cells was fractionated on a 1% agarose gel (33), transferred to a nylon membrane (51), and cut into four pieces. Each membrane was hybridized as described above with each of four probes, the genomic *Hin*cII-*Hin*dIII fragment containing the *RHA1* sequence, *Sal*I-*Pst*I fragments of pJK-Sp1-14 and pJK-Sp2-14, and the *Eco*RI-derived fragment of cDNA 14. Probes were radiolabeled with random oligonucleotides (16). The membrane-washing condition was slightly modified. After washing the membrane at room temperature in 2× SSC–0.1% SDS, rewashing in 0.1× SSC–0.1% SDS was performed twice at 42°C, twice at 52°C, and then twice at 62°C, each for 15 min.

In each temperature-raising step, cross-hybridization was observed by autoradiography, and it disappeared in the last washing condition.

Synthetic peptide and preparation of antipeptide antibody. A peptide of the sequence H-Tyr-Pro-Glu-Ile-Ser-Trp-Thr-Arg-Asn-Gly-Cys-Thr-Val-Ser-OH was synthesized by the solid-phase procedure of Merrifield (32) and was purified by Sephadex G-10 column chromatography. The synthetic peptide contains the peptide moiety of rhodotorucine *A* and three additional amino acids at the carboxy terminus derived from a spacer sequence. This peptide was coupled to carrier protein keyhole limpet hemocyanin through the cysteine residue of the peptide by using *m*-maleimidebenzoyl-*N*-hydroxysuccinimide ester as the coupling reagent, as described by Lerner et al. (30). Rabbits were immunized six times at 2-week intervals with the peptide-coupled keyhole limpet hemocyanin as described previously (52). The activity of antibodies was measured by the enzyme-linked immunosorbent assay method as recommended by the supplier (Zymed Laboratories).

Immunoprecipitation of in vitro translation products. In vitro translation reactions were performed with a nuclease-treated wheat germ extract by following the recommendations of the supplier (DuPont, NEN Research Products). The reaction mixture (25 μl) contained 50 μCi of [^{35}S]methionine (Amersham Corp.) and 5 μg of poly(A)$^+$ RNA isolated from *A* cells of *R. toruloides*. The concentrations of the other components of the reaction mixture were as specified by the manufacturer. Incubations were conducted for 120 min at 22°C. For immunoprecipitation, *Staphylococcus aureus* cells were adsorbed with antibodies. A 50-μl portion of a 10% (vol/vol) suspension of fixed *S. aureus* cells in immunoprecipitation buffer II (IPB-II) (50 mM Tris hydrochloride [pH 8.2], 0.15 M NaCl, 5 mM EDTA, 1% [vol/vol] Triton X-100, 0.2% [wt/vol] SDS) was mixed either with 75 μl of antipeptide antiserum or nonimmune serum. It was incubated at 0°C for 1 h, centrifuged at 8,000 × *g* for 1 min, washed once with IPB-II, and resuspended in 50 μl of IPB-II. A sample (20 μl) of in vitro translation mixture was mixed with 30 μl of IPB-II. This mixture was added with 50 μl of 10% (vol/vol) suspension of *S. aureus* cells in IPB-II, incubated at 0°C for 30 min, and centrifuged at 8,000 × *g* for 1 min. To the supernatant fluid, an above-mentioned suspension (50 μl) of *S. aureus* cells adsorbed with antibodies was added. For the competitive reaction with antigen peptide, 100 μg of synthetic peptide was added to this mixture. The reaction mixtures were incubated at 0°C overnight, and cells adsorbed with immune complexes were collected by centrifugation. The pelleted cells were washed three times with 0.5 ml of IPB-III, which was the same as IPB-II except that concentrations of Triton X-100 and SDS were reduced 10-fold, and suspended in 20 μl of sample buffer (10 M urea, 1.25% [vol/vol] 2-mercaptoethanol, 1.14% [wt/vol] SDS, 12.5 mM H_3PO_4 adjusted to pH 6.8 with Tris). The adsorbed immune complexes were solubilized by keeping them in a boiling water bath for 3 min. They were clarified by centrifugation at 8,000 × *g* for 10 min, and then the supernatants were loaded in the gel wells. Gel electrophoresis was performed by the procedure of Swank and Munkres (50) in SDS-urea-polyacrylamide (7.5%) slab gel. Fluorography of polyacrylamide gels, after impregnation with salicylate (11), was conducted at −70°C.

RESULTS

Cloning and sequencing of cDNAs encoding putative rhodotorucine *A* precursors. Previously, a gene coding for the peptide moiety of rhodotorucine *A*, *RHA1*, has been cloned by using synthetic oligonucleotides as probes (2). The restriction map of *RHA1* is shown in Fig. 1. This gene was used to isolate cDNAs encoding pheromone precursors. A complementary DNA library in λgt10 was produced by oligo(dT) priming of total poly(A)$^+$ RNA. Screening of this library allowed the identification of 15 positive clones. Complete DNA sequences of four clones (T, 26, A and R) were determined (Fig. 2). These four cDNA clones were classified into two types. Two cDNAs (clones T and 26) had almost identical sequences with *RHA1*, and the other two (clones A and R) were similar to *RHA1* but had different sequences. There was one difference between two of the clones, 26 and T, that led to a single conservative amino acid substitution. In the other two clones, A and R, there were five nucleotide substitutions, one silent substitution in the open reading frame and four substitutions 3′ to the coding region. Comparisons to the pheromone-coding frame showed that the cDNA inserts contained open reading frames of 62 amino acids (clones 26 and T) or 77 amino acids (clones A and R).

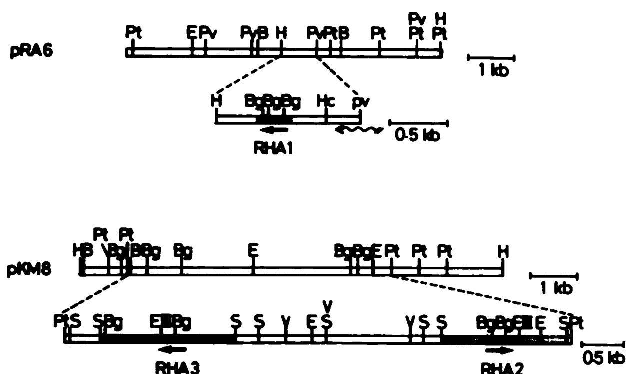

FIG. 1. Restriction maps of rhodotorucine *A* genomic clones. Three genes named *RHA1*, *RHA2*, and *RHA3* are present. Arrows show the direction of transcription of these genes. The open reading frames of the rhodotorucine *A* genes are shown by closed boxes. Two *Sal*I fragments hybridized with the probe (cDNA clone 75) are indicated by shaded boxes. A wavy line with an arrow shows the site of mRNA that cloned as cDNA 14, which did not encode pheromone precursor sequence and was used in Northern blot analysis as a control. Symbols for restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; EIII, *Eco*47III; H, *Hin*dIII; Hc, *Hin*cII; Pt, *Pst*I; Pv, *Pvu*II; S, *Sal*I; V, *Eco*RV.

Cloning and sequencing of additional *RHA* genes. Since Southern blot analysis with the previously identified *RHA1* gene identified a second hybridizing fragment of 9 kb (data not shown), a size-selected DNA library constructed by inserting *Hin*dIII-digested genomic DNA fragments of about 9 kb into pBR322 was screened by using cDNA clone 75 as a probe. From isolated clones having 9-kb inserts flanked by *Hin*dIII sites, one (pKM8) was selected and mapped by using restriction enzymes (Fig. 1). Southern blot analysis of pKM8 showed that two regions (indicated by shaded bars in Fig. 1) hybridized with the probe (data not shown). Sequence analysis showed that one region (*RHA2*) encoded five copies of the peptide moiety of rhodotorucine *A* and another region (*RHA3*) encoded three copies. The nucleotide and predicted amino acid sequences of the three rhodotorucine *A* genes are shown in Fig. 3. All three genes had tandem repeat sequences encoding the pheromone peptide. The numbers of repeat units were 4 in *RHA1*, 5 in *RHA2*, and 3 in *RHA3*.

A

```
                                                 Clone T     /////
                                                 Clone 26    CTTTC

        -40                 -20                  1
                                                 met val ala
T   ////////CCTCTCCAAACACCCGCCTACTCTCCACACTTCAACAAC ATG GTC GCC
26  GCCTTCCT--------------------------------------- --- --- ---

                20                          40
    tyr pro glu ile ser trp thr arg asn gly cys thr val ser lys
T   TAC CCC GAG ATC TCC TGG ACC CGC AAC GGC TGC ACC GTC TCC AAG
26  --- --- --T --- --- --- --- --- --- --- --- --- --- --- ---
            asp
        60                          80
    tyr pro glu ile ser trp thr arg asn gly cys thr val ser lys
T   TAC CCG GAG ATT TCG TGG ACC CGC AAC GGC TGC ACC GTC TCC AAG
26  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

    100                     120                         140
    tyr pro glu ile ser trp thr arg asn gly cys thr val ser lys
T   TAC CCC GAG ATC TCG TGG ACC CGC AAC GGT TGC ACC GTC TCC AAG
26  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                        160                         180
    tyr pro glu ile ser trp thr arg asn gly cys thr val ala END
T   TAC CCG GAG ATC TCG TGG ACC CGC AAC GGT TGC ACC GTC GCC TAA
26  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

              200                 220                 240
T   TCCTCCCTCAACCAATCCATCCGCCCCGCTCGCCTACCCTCTCCTCCTTCATTGTCGTT
26  -----▲-----------------------------------------------------

              260                 280                 300
T   CGTCGTCAAAGCAGGAACTGGTCGGTGGAACGGGTACGGGATCAAGGAGGAGGAAGAAG
26  -----------------------------------------------------------

               320                 340                 360
T   GAGGGGTGTTTGAGGAGGGTATCTTTATTCGCGCAAGCTTGCATCGTCGTCCACACTCG
26  -----------------------------------------------------------

T   TTTTATACCGTC(A)n
26  ------------(A)n
```

B

```
                                                 Clone A    CTTTC
                                                 Clone R    -----

        -40                 -20                  1
                                                 met val ala
A   GCCTTCCTCCTCTCCAAACACCCGCCTACTCTCCACTCTTCAACAAC ATG GTC GCC
R   ----------------------------------------------- --- --- ---

                20                          40
    tyr pro glu ile ser trp thr arg asn gly cys thr val ala lys
A   TAC CCC GAG ATC TCC TGG ACC CGC AAC GGC TGC ACC GTC GCC AAG
R   --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

        60                          80
    tyr pro glu ile ser trp thr arg asn gly cys thr val ala lys
A   TAC CCC GAA ATC TCG TGG ACC CGC AAC GGC TGC ACC GTC TCC AAG
R   --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

    100                     120                         140
    tyr pro glu ile ser trp thr arg asn gly cys thr val ala lys
A   TAC CCG GAG ATT TCG TGG ACC CGC AAC GGC TGC ACC GTC TCC AAG
R   --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                        160                         180
    tyr pro glu ile ser trp thr arg asn gly cys thr val ala lys
A   TAC CCC GAG ATC TCG TGG ACT CGC AAC GGT TGC ACC GTC TCC AAG
R   --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                200                         220
    tyr pro glu ile ser trp thr arg asn gly cys thr val ala END
A   TAC CCG GAA ATC TCG TGG ACC CGC AAC GGT TGC ACC GTC GCC TAA
R   --- --- --G --- --- --- --- --- --- --- --- --- --- --- ---

        240                 260                 280
A   TCCTCCCCCGCCGCATCTCAATCGCTCGGATTCCCTGTCATCACTCGCCGTCGCAAGCG
R   ------▲----------------------------------------------------

         300                 320                 340
A   CTTCAAGGCAAGGACCGGTCAAGCCATCTCCGAGGACGCTGCGAGGAGGATCTGGGGCG
R   -------------T----------------G-------------------T--------

          360                 380
A   AGCGTCGTATCTTTATTGGTGTTTTGCAATTGTCGTTCCTCGAT(A)n
R   ------------------------------------/------G-(A)n
```

FIG. 2. Nucleotide and deduced amino acid sequences of cDNA clones. –, Identical sequences; /, deletion. The numbers above denote the positions of nucleotides. (A) cDNA clones homologous to genomic *RHA1*. Clones T and 26 have a different base at position 18. (B) Other cDNA clones (*RHA2*-type). Two clones, A and R, have five base changes, and clone R has one deletion. The peptide sequence of rhodotorucine *A* are indicated in bold type. Identical sequences, GTATCTTTATT, at the 3′ noncoding regions of all clones are overlined. Arrowheads show intron splice sites. $(A)_n$, Poly(A) sequence.

Nucleotide sequences of *RHA2* and *RHA3* are highly similar to each other, except for the number of tandem repeats and the last 30 nucleotides of the 3′ end (Fig. 3). *RHA1* had no sequence similarity to *RHA2-RHA3* at the 5′ upstream region from −192 and at the 3′ noncoding downstream region. An identical 11-bp sequence was present in the 3′ noncoding regions of all three genes, although there is no consensus sequence for the polyadenylation signal, such as AATAAA (7).

Comparisons of nucleotide sequences between genomic *RHA* and cDNAs revealed that *RHA1* and *RHA2* were matched to cDNA clones T and R and that each gene contained introns of 60 bp and 67 bp at the 3′ noncoding regions, respectively. The introns possessed a consensus sequence, GT – – – CT(A/G)AC – – – AG, that may be important for splicing. At the 5′ upstream noncoding regions, several repeat sequences of C(C/G)(T/A/C)TTGT were present.

Predicted precursor structure of rhodotorucine *A*. From the open reading frames of the three genes, three precursors for rhodotorucine *A* were predicted. *RHA1*, *RHA2*, and *RHA3* encoded 62 amino acids (calculated molecular weight [M_W] 7,067), 77 amino acids (M_W 8,774), and 47 amino acids (M_W 5,328), respectively. These three putative precursors were similar to each other except for the number of tandem repeats with similar leaders, spacers, and carboxy-terminal sequences. The leader sequences preceding the tandem repeats of the pheromone peptide contained only three amino acids. In addition, no large stretches of hydrophobic amino acids were found elsewhere. The amino-terminal three amino acids and the following sequences do not show the features of hydrophobic signal sequences nor sites for Asn-linked glycosylation usually found in precursors of secreted proteins.

Determination of transcription start sites. To verify our assignment of the initiator methionine, we searched for the transcriptional start site by primer extension and S1 nuclease protection methods. An 18-mer primer complementary to the sequence at the 5′ upstream region (−9 to 9) of the pheromone-coding sequence was chemically synthesized. The radiolabeled 18-mer primer was annealed to poly(A)$^+$ RNAs, and cDNAs were produced by reverse transcriptase. For S1 protection, a single-stranded DNA probe derived from genomic *RHA1* was hybridized with poly(A)$^+$ RNAs and the size of fragment protected from S1 nuclease digestion was analyzed. The products of primer extension and those of S1 protection agreed well in length (Fig. 4). All three *RHA* genes contained the 18-mer primer sequence, and they had very similar 5′ upstream sequences from −1 to −191. In addition, *RHA* mRNAs were transcribed as revealed by Northern blot analysis (see below). Therefore, the major and minor bands seen in Fig. 4 represent start sites of the three types of *RHA* mRNAs, although the precise start sites for each of the *RHA* mRNAs were not clear from this experiment. From the transcriptional start sites to the pheromone coding region, only one initiator codon (ATG) was present,

```
                                    RHA1      GCTCTCGCCGAAG -228
                                    RHA2      CGAGGGCCTTTGT -228
                                    RHA3      CGAGGGCCTTTGT -228

RHA1 TCTTCGTCCTTTGTGGGTGTGTCCCGGTCCGATTCCCGTCTGCCTTTGTTCGTCGATGCT -168
RHA2 TCTTGTCGTCTGCCCTTTGTTCCATGATCGTTTGTTCGTCTGCGTTTGTTCGTCGATGCT -168
RHA3 TCTTGTCGTCTGXCCTTTGTTCCATGATCGTTTGTTCGTCTGCGTTTGTTCGTCGATGCT -168

RHA1 GACCTTTGTCCATTGTGCAGGCTTGTCTTGTCGTCTCCCCTTGTTCTCGGTGGCTATTTG -108
RHA2 GACCTTTGTCCATTGTGCAGGCTTGTCTTGTCGTCTCCCCTTGTTCTCGGTGGCTATTTG -108
RHA3 GAXCTTTGTCCATTGTGCAGGCTTGTCTTGTCGTCTCCCCTTGTTCTCGGTGGCTATTTG -108

RHA1 AGAGCCGCTCGTCGTTTCGACGCTCTCTCCTTCCTACATCCCTCCTCAGCCAACTCTTTC -48
RHA2 AGAGCCGCTCGTCGTTTCGACGCTCTCTCCTTCCTACATCCCTCCTCAGCCAACTCTTTC -48
RHA3 AGAGCCGCTGCTCGTTTCGACGCTCTCTCCTTCCTACATCCCTCCTCAGCCAACTCTTTC -48
                                                   1
                                                   met val ala
RHA1 GCCTTCCTCCTCTCCAAACACCCGCCTACTCTCCACACTTCAACAAC ATG GTC GCC  9
RHA2 GCCTTCCTCCTCTCCAAACACCCGCCTACTCTCCACTCTTCAACAAC ATG GTC GCC  9
RHA3 GCCTTCCTCCTCTCCAAACACCCGCCTACTCTCCACTCTTCAACAAC ATG GTC GCC  9
                                                       ala
     tyr pro glu ile ser trp thr arg asn gly cys thr val ser lys
RHA1 TAC CCC GAG ATC TCC TGG ACC CGC AAC GGC TGC ACC GTC TCC AAG  54
RHA2 TAC CCC GAG ATC TCC TGG ACC CGC AAC GGC TGC ACC GTC GCC AAG  54
RHA3 TAC CCC GAG ATC TCC TGG ACC CGC AAC GGC TGC ACC GTC GCC AAG  54

     tyr pro glu ile ser trp thr arg asn gly cys thr val ser lys
RHA1 TAC CCG GAG ATT TCG TGG ACC CGC AAC GGC TGC ACC GTC TCC AAG  99
RHA2 TAC CCC GAA ATC TCG TGG ACC CGC AAC GGC TGC ACC GTC TCC AAG  99
RHA3 TAC CCC GAA ATC TCG TGG ACC CGC AAC GGC TGC ACC GTC TCC AAG  99

     tyr pro glu ile ser trp thr arg asn gly cys thr val ser lys
RHA1 TAC CCC GAG ATC TCG TGG ACC CGC AAC GGT TGC ACC GTC TCC AAG  144
RHA2 TAC CCG GAG ATT TCG TGG ACC CGC AAC GGC TGC ACC GTC TCC AAG  144
RHA3 /// /// /// /// /// /// /// /// /// /// /// /// /// /// ///

     tyr pro glu ile ser trp thr arg asn gly cys thr val ser lys
RHA1 /// /// /// /// /// /// /// /// /// /// /// /// /// /// ///
RHA2 TAC CCC GAG ATC TCG TGG ACT CGC AAC GGT TGC ACC GTC TCC AAG  189
RHA3 /// /// /// /// /// /// /// /// /// /// /// /// /// /// ///

     tyr pro glu ile ser trp thr arg asn gly cys thr val ala END
RHA1 TAC CCG GAG ATC TCG TGG ACC CGC AAC GGT TGC ACC GTC GCC TAA  189
RHA2 TAC CCG GAG ATC TCG TGG ACC CGC AAC GGT TGC ACC GTC GCC TAA  234
RHA3 TAC CCG GAA ATC TCG TGG ACC CGC AAC GGT TGC ACC GTC GCC TAA  144

RHA1 TCCTCCgttcgtcccgccctacctcacatcaccgcgcatgcccgctaacgctcctccccc 249
RHA2 TCCTCCgtgagtcctcctctttctcctgagtcgcgactggcagaacctgacgcccttgca 294
RHA3 TCCTCCGTGAGTCCTCCTCTTTCTCCTGAGTCGCGACTGGCAGAACCTGACACCCTTGCA 204

RHA1 tcgcagCTCAACCAATCCATCCGCCCCGCTCGCCTACCCTCTCCTCCTTCATTGTCGTTC 309
RHA2 tgcattctcgcagCCCGCCGCATCTCAATCGCTCGGATTCCCTGTCATCACTCGCCGTCG 354
RHA3 TGCATTCTCGCAGCCCGCCGCATCTCAATCGCTCGGATTCCCTGTCATCACTCGCCGTCG 264

RHA1 GTCGTCAAAGCAGGAACTGGTCGGTGGAACGGGTACGGGATCAAGGAGGAGGAAGAAGGA 369
RHA2 CAAGCGCTTCAAGGCAAGGTCCGGTCAAGCCATCTCGGAGGACGCTGCGAGGAGGTTCTG 414
RHA3 CAAGCGCTTCAAGGCAAGGACCGGTCAAGCCATCTCCGAGGACGCTGCGAGGAGGATCTG 324

RHA1 GGGGTGTTTGAGGAGGGTATCTTTATTCGCGCAAGCTTGCATCGTCGTCCACACTCGTTT 429
RHA2 GGGCGAGCGTCGTATCTTTATTGGTGTTTTGCAATTGTCGT/CCTCGGTACCTCTCGCCG 473
RHA3 GGGCGAGCGTCGTATCTTTATTGGTGTTTTGCAATTGTCGTTCCTCGATACCTCTCGCCG 384

RHA1 TATACCGTCTTTCGAGCGCCATATACGCGCCATATCCGAGAGAACTTGTTGAAGG      484
RHA2 CCAGAACGCGAGCCTCAGCCGATGCGAGCGGGATGCTGCCTGTCCTCGATTAGCAAGTCT 533
RHA3 CCCGAACGCGAGCGTCAGCCAATGCGAGCACCGCGGACCGGACGTTGAGAAGTCCTGCTT 444
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FIG. 3. Comparison of the nucleotide sequences of three rhodotorucine *A* genes, *RHA1*, *RHA2*, and *RHA3*. The proposed coding regions are shown with the corresponding amino acids above. Deletion is indicated by /. Nucleotides are numbered from the initiation codon. Repeated sequences located in the 5′ noncoding region are marked by underlines. mRNA start sites are indicated by arrows. Introns are indicated by lowercase letters, in which intron consensus sequences are indicated by over and underlines. Intron of *RHA3* has not been identified, but intron consensus sequences are also marked. Identical 11-bp sequences at the 3′ noncoding regions are indicated by overlines. X indicates an unidentified nucleotide.

and it was in phase with the pheromone reading frame. Additionally, the flanking sequence of this ATG contained a conserved sequence proposed by Kozak (28), i.e. TCA(C/A)(A/C)*AUG*(G/U)C, found in filamentous fungi (4). These results confirmed the primary precursor structure of rhodotorucine *A* deduced from the sequence analyses.

Northern blot analysis of *RHA* mRNAs. The transcripts of the three types of *RHA* genes were examined. For this purpose, we constructed specific probes (pJK-Sp1-14 and pJK-Sp2-14) designed to specifically detect mRNAs derived from the *RHA*-type and *RHA2-RHA3*-type genes. By using four probes, genomic *RHA1* DNA, pJK-Sp1-14, pJK-Sp2-14, and cDNA 14, Northern blot analysis was performed (Fig. 5). When genomic *RHA1* DNA (*Hin*cII-*Pvu*II fragment containing the pheromone-coding sequence) was used as a probe, a thick band of 0.65 to 0.8 kb was seen on the blots. Since this genomic probe can hybridize with all three types of *RHA* genes (data not shown), this band represented a

FIG. 4. Primer extension and S1 nuclease protection analyses. The products extended by reverse transcriptase (cDNA) and protected from S1 nuclease digestion (S1) are shown at the top. These products were fractionated in the sequence gel electrophoresis as described in the text. CGTA displays a sequence ladder of genomic *RHA1* DNA extended from synthetic 18-mer primer, which was used as a size marker. Nucleotide sequence of 5′ upstream region of *RHA1* DNA is shown at the bottom. Sequence complementary to the 18-mer primer is underlined. Asterisks indicate the mRNA start sites determined.

mixture of mRNAs of the three types of *RHA* genes. When the specific probes were used, two bands were seen. A larger 1.2-kb band was due to the control cDNA 14 probe, which did not encode the pheromone precursor sequence (Fig. 1). The *RHA1*-specific probe detected a 0.7-kb narrow band, while the *RHA2-RHA3*-specific probe detected a broad band(s) of 0.65 to 0.8 kb. The mRNAs of *RHA2* and *RHA3* could not be distinguished by this probe. However, the high sequence homology between *RHA2* and *RHA3* and the tandemly repeated structure of the coding sequence allowed us to speculate that the size difference of the two types of

FIG. 5. Northern blot analysis. Poly(A)$^+$ RNA isolated from *A* cells of *R. toruloides* was fractionated in a 1% agarose gel, transferred to a nylon membrane, and hybridized with four probes as described in the text; pJK-Sp1-14 and pJK-Sp2-14 contain nonhomologous 3′ noncoding regions of *RHA1*-type and *RHA2*-type cDNAs, respectively. Size markers, which were prepared from pRA6 by treatment with glyoxal, are indicated at the left. Arrow indicates bands of *RHA* mRNAs (0.65 to 0.8 kb) and arrowhead indicates a band of cDNA 14 (1.2 kb).

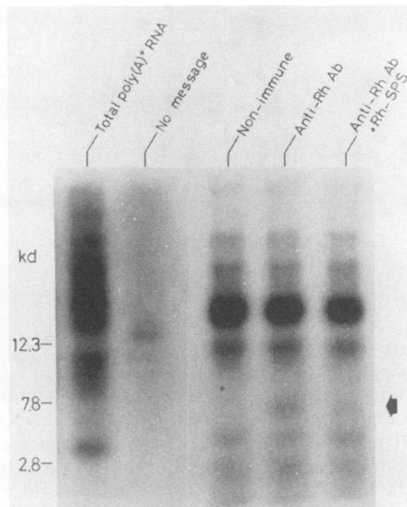

FIG. 6. Analysis of rhodotorucine *A* precursor protein by immunoprecipitation of in vitro translation products. In vitro translation was performed in the presence [Total poly(A)$^+$ RNA] and the absence (No message) of poly(A)$^+$ RNAs prepared from *A* cells of *R. toruloides*. Radiolabeled proteins antigenically related to rhodotorucine *A* were immunoprecipitated, subjected to electrophoresis in a slab of SDS-urea polyacrylamide gel, and examined by fluorography as described in the text. Total material immunoprecipitated with nonimmune serum (Non-immune), antipeptide antiserum (Anti-Rh Ab), and antiserum containing 100 μg of antigen peptide (Anti-Rh Ab + Rh-SPS) were loaded in the gel wells. Arrow indicates the material specifically immunoprecipitated by the antipeptide antiserum. Molecular weight markers are cytochrome *c* (12.3 kilodaltons [kd]) and its cyanogen bromide cleaved fragments (7.8 and 2.8 kDa).

mRNA species may correspond to the difference in the number of the repeated coding sequences of the genes. Since one stretch of the coding sequence of the rhodotorucine *A* peptide plus a spacer peptide was 45 bp, the sizes of the *RHA2* and *RHA3* mRNAs may differ by 90 bp. The band width estimated by Northern blot analysis agreed with this calculated size difference. Thus, the broad band probably contained mRNAs of both *RHA2* and *RHA3*. However, as we have no cDNA sequence corresponding to *RHA3*, there is no clear evidence that *RHA3* is expressed.

Immunological detection of rhodotorucine *A* precursor. To test for a precursor of the predicted size, an immunological method was used. Antibody was prepared against a synthetic tetradecapeptide consisting of the pheromone peptide and spacer peptide sequence. We performed immunoprecipitation of ^{35}S-labeled protein products translated by an in vitro system of wheat germ extract with total poly(A)$^+$ RNA by using the antiserum. Specific protein product(s) was detected in SDS polyacrylamide gels as specific material(s) immunoprecipitated by the antiserum, but not by the nonimmune serum (Fig. 6). When the immunoprecipitation was performed with antiserum together with the peptide antigen, the intensity of the specific proteins, but not of the others, was diminished. This suggests that the radioactive-specific product(s) competes with the peptide antigen for binding to the antibody. Based on the mobility relative to a protein standard of known M_W, the specific protein product(s) detected had a M_W of about 7,000. We have predicted three precursors of rhodotorucine *A* having M_W of 5,328, 7,067, and 8,774. The size of the immunologically specific material(s) agrees with that of the predicted precursor deduced from *RHA1*. It is not known why the other precursor bands were not detected by this experiment, though the mRNAs of three *RHA* genes are present, as revealed by the Northern blot analysis. The following reasons are assumed: (i) the gel electrophoresis system used could not separate the three precursors, (ii) nonrhodotorucine *A*-related peptides present in all lanes could have obscured one or more species, or (iii) the various mRNA species could be translated at widely varying efficiencies, so *RHA2-RHA3*-type mRNAs could not be detected.

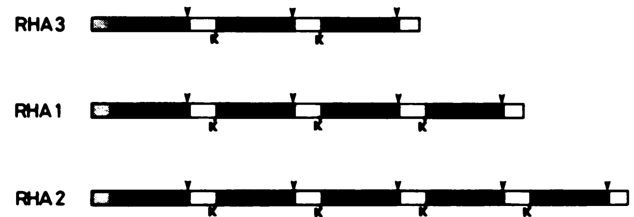

FIG. 7. Schematic representation of the three rhodotorucine *A* precursors. Closed box shows the peptide moiety of rhodotorucine *A*, open box shows the spacer peptide, and shaded box shows the short leader peptide Met-Val-Ala. Arrowhead indicates the site of cysteine attached with farnesyl residue and K indicates a single basic residue, lysine, potentially used as processing site.

DISCUSSION

In the present study, we isolated and sequenced cDNAs and genomic DNAs encoding the peptide moiety of rhodotorucine *A*, and we deduced three primary precursor structures of rhodotorucine *A*. The precursor structures are illustrated in schematic form in Fig. 7. By using genomic *RHA1* DNA as a probe, several cDNA clones were obtained. Moreover, by using a cDNA clone as a probe, a genomic 9-kb *Hin*dIII fragment was cloned. Surprisingly, this fragment contained two genes for distinct rhodotorucine *A* precursors.

Nucleotide sequence analyses of cDNA clones revealed that two cDNA clones (T and 26) were homologous to *RHA1* and two others (A and R) were homologous to *RHA2*. One and six base changes were found between T and 26 and between A and R, respectively. Genomic Southern blot analyses reported previously (2) indicated that the sizes of detected bands agreed precisely with the map of the cloned genomic DNA and showed that other bands were not present. In cDNA clones T and 26, one substitution (nucleotide number 18) leads to the prediction of restriction enzyme polymorphism. Here, an *Eco*RV site is present in clone 26 but not in clone T. Genomic Southern blot analysis with *Eco*RV and pJK-Sp1 probes suggested that a genomic DNA fragment possessing this *Eco*RV site of clone 26 was not present (data not shown). Therefore, these base changes may have occurred during the cloning procedures. Since we used an amplified cDNA library for the screening, multiple identical DNA clones may result from the isolation of sibling phage.

The predicted rhodotorucine *A* precursors contained multiple tandem copies of the pheromone peptides. *RHA3*, *RHA1*, and *RHA2* contained three, four, and five copies, respectively. Such a repeated arrangement has been also observed in α-factor precursors of several *Saccharomyces* species (9) and in the neuropeptide FMRFamide (44). The generation of these multiple repeats could be explained by an unequal crossing-over mechanism (49). The multiple peptides per mole of precursor and multiple genes in a genome

may increase the production of the mating pheromone per cell, which may be a prerequisite for the diffusible small peptides to function effectively.

It is well known that precursors of secretory proteins generally contain 15 to 30 additional amino acids, predominantly hydrophobic ones, at the amino termini, and these are called signal sequences. They serve as signals for membrane translocation and target proteins for the classical secretory pathway. With rhodotorucine *A* precursors, the amino-terminal sequence Met-Val-Ala is hydrophobic, but this sequence may not function as a signal sequence because of its short length. In addition, there are no other hydrophobic stretches elsewhere in the precursors. We tentatively conclude that the rhodotorucine *A* precursors do not contain a classical signal sequence. A few other secretory proteins known not to contain signal sequences include the precursors of FMRFamide (44), interleukin-1 (31), and **a**-factor (8). Little is known about the secretory mechanism for these proteins.

With rhodotorucine *A*, modification by the addition of the nonpolar polyisoprenyl residue may play a major role in secretion in place of the classical signal sequence. Recently, fatty acid acylations have been recognized to be important for the targeting of several proteins (45). Among these are the *ras* and *src* proteins that regulate cell proliferation. These also do not have signal sequences, yet are localized to the plasma membrane. It is suggested that the membrane targeting of these proteins uses a novel pathway distinct from the classical secretory pathway and that fatty acid acylation plays an important role in this process (27, 53). In *ras* proteins, palmitic acid is attached via a thioester bond to the cysteine residue located near the carboxy terminus of the protein. The sequence Cys-A-A-X (A is an aliphatic amino acid and X is the carboxy-terminal amino acid) is present at the carboxy terminus of the proteins and is conserved in all eucaryotic *ras* proteins (38). The spacer sequence (Cys)-Thr-Val-Ser/Ala of the rhodotorucine *A* precursors is similar to this conserved sequence. In rhodotorucine *A*, a farnesyl moiety is attached via a thioether bond to the cysteine residue. The spacer sequence next to the cysteine residue may play a signaling role for the addition of the farnesyl residue. From studies of tremerogen *A*-10, a polyisoprenylated peptide mating pheromone of another basidiomycetous yeast, the requirement of polyisoprenylation for pheromone secretion has been suggested (34).

Recently, the structure of mating pheromone **a**-factor of *S. cerevisiae* has been identified (3, 6). **a**-factor contains 12 amino acids, and its carboxy-terminal cysteine is modified by the addition of a farnesyl residue identical to those of the mating pheromones of the basidiomycetous yeasts. The **a**-factor precursor also does not have a signal sequence but has the common carboxy-terminal sequence identical to that of the *ras* proteins (8). Mutations (*dprl* or *ram*) causing defects in the processing of *RAS* protein affect the production of **a**-factor (17, 38). From these results, it was suggested that the secretion of **a**-factor uses a pathway in common with that of *RAS* proteins. Thus, the result presented in this study suggest that rhodotorucine *A* precursors use a secretory pathway similar to that of **a**-factor and *RAS* protein.

The precursor structure of rhodotorucine *A* suggests other unique processing steps different from those of other peptide hormones. Generally, pairs of basic amino acids are known as the recognition sites for proteolytic enzymes to produce mature hormones (19). In contrast, rhodotorucine *A* precursors do not have pairs of basic amino acids but have a single basic amino acid, lysine. This lysine occurs at the amino-terminal side of each pheromone peptide repeat, except for the first repeat (Fig. 7). Thus, it must be cleaved for the maturation of the pheromone. It is known that polypeptide precursors having a monobasic processing site are rare and that such a basic amino acid is usually arginine (47). To our knowledge, monobasic lysine-directed cleavage is known only for precursors of FMRFamide (44) and antrin (5). Spacer peptides presumably attached to the carboxy terminus of the pheromone peptide are also removed by an unknown mechanism. Thus, novel proteolytic processing steps must be involved in the maturation for rhodotorucine *A*.

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