A Mutation in the Signal Recognition Particle 7S RNA of the Yeast Yarrowia lipolytica Preferentially Affects Synthesis of the Alkaline Extracellular Protease: In vivo Evidence for Translational Arrest

Debbie S. Yaver, Sam Matoba,* and David M. Ogrydziak*
Department of Microbiology, University of California, Davis, California 95616; and *Institute of Marine Resources, University of California, Davis, California 95616

Abstract. Replacement of the signal recognition particle (SRP) 7S gene (SCRI) on a replicating plasmid with scr1-l (G to A at 129 and A to T at 131 in the consensus sequence -GNAR- in the loop of domain III) resulted in temperature sensitivity for growth of cells in which both chromosomal SRP 7S RNA genes were deleted. Pulse-chase immunoprecipitation experiments were done after a shift to non-permissive temperature using the major secreted protein the alkaline extracellular protease (AEP) as a reporter molecule. No untranslocated AEP precursor was detected in a strain with scr1-l on a plasmid, but the amount of the largest AEP precursor (55 kD) immunoprecipitated as a percentage of total proteins synthesized was reduced 68% compared to an isogenic strain with SCR1 on the plasmid. The possibility that an untranslocated precursor was synthesized but not detected because of instability was largely eliminated by detection of a 53-kD untranslocated precursor of a mutated AEP (P17M; methionine replaced proline in the second position of the pro-peptide) which chased to the 55-kD translocated AEP precursor. Thus, SRP has a role in the biosynthesis of AEP. Possibly, the scr1-l mutation does not affect signal recognition or translational arrest but instead results in maintenance of translational arrest of AEP synthesis. The results also suggest that AEP can be translocated in vivo either co-translationally in which SRP is at least involved in biosynthesis or posttranslationally without SRP involvement.

In higher eukaryotes, evidence suggests that the signal recognition particle (SRP) is essential for protein translocation across the ER membrane. SRP is a soluble 11S ribonucleoprotein composed of six polypeptides (72, 68, 54, 19, 14, and 9 kD) and a single 7S RNA of 300 nucleotides (66, 74). From the study of in vitro systems, a model has been proposed in which SRP functions as an adapter between the translational machinery in the cytoplasm and the translocational machinery in the ER membrane (49, 66, 74). The functions of individual SRP proteins have been elucidated by the study of "mutant" SRPs reconstituted in vitro (63, 65). Three functions of SRP have been identified: signal recognition, elongation arrest, and translocation promotion (66). Translation of mRNAs coding for secretory proteins begins on free ribosomes in the cytoplasm. When the polypeptide chain has elongated sufficiently, SRP binds the signal sequence/ribosome complex. This interaction results in arrest or pausing of translation (74, 78). The SRP/ribosome complex is then targeted to the SRP receptor, an ER integral membrane protein. This results in the release of SRP and translational arrest, and the polypeptide is then co-translationally translocated into the ER. During or shortly after translocation, signal peptide cleavage and core glycosylation occur. Besides a role as a scaffold for binding SRP proteins (73), functional roles for SRP 7S RNA in elongation arrest and in interaction with the SRP receptor have been proposed (36, 63, 81). Recently, it has been shown that SRP 7S RNA undergoes conformational changes which possibly are a necessary component for movement through the SRP cycle (3).

The study of the in vivo functions of SRP was made possible by the isolation of SRP homologues and SRP 7S RNA genes in two genetically tractable organisms, Yarrowia lipolytica (30, 46) and Schizosaccharomyces pombe (12, 46, 51). The yeast RNAs resemble higher eukaryotic 7S RNA with respect to size, transcriptional start signals, 5' end structures, potential secondary structures, and binding under stringent conditions to mammalian SRP proteins. S. pombe contains a single essential SRP 7S RNA (12, 51). Mutations in a conserved tetranucleotide loop in domain IV demonstrated it is important for function; several of the mutations resulted in osmotic sensitivity at higher temperatures (37).

Homologues to Srp54p have been isolated from Saccharomyces cerevisiae and S. pombe (1, 26). Antibodies against Srp54p from S. pombe coprecipitate the S. pombe 7S RNA, and antibodies against Srp54p from S. cerevisiae coprecipitate S. cerevisiae SCRI RNA (519 nucleotides).
Deletion of 252 nucleotides from the SCRI coding region results in sick but viable cells (22). Recent studies in E. coli, although controversial (7, 8, 13, 48), suggest that a ribonucleoprotein containing the 4.5S RNA has SRP-like properties (47, 52) and that protein secretion can occur by posttranslational and SRP-like homologue mediated co-translational pathways (47, 50, 52).

In this study we isolated a temperature sensitive mutation in one of the genes (SCRI) coding for the Y. lipolytica SRP 7S RNA. Y. lipolytica is a dimorphic, heterothallic yeast which is quite different from S. cerevisiae (5) and S. pombe. Y. lipolytica contains two functional SRP 7S RNA genes: SCRI and SCRII (31). Disruption of either gene alone has no obvious effect on growth or secretion, but disruption of both genes is lethal (31). Y. lipolytica secretes significant levels of several hydrolytic enzymes, and it produces an alkaline extracellular protease (AEP) at levels of 1 to 2% of total cell protein (40, 43). AEP processing involves several intracellular precursors; the largest and earliest precursor detected in pulse-chase immunoprecipitation experiments is a 55-kD translocated polypeptide which lacks the signal peptide (21, unpublished data) but contains 2 kD of N-linked carbohydrate (39, 40). The next largest AEP precursor is a 52-kD polypeptide which results from dipeptidyl aminopeptidase processing (39). The major processing pathway is from the 55- to the 52-kD precursor and then to the 32-kD mature AEP (40). In wild type strains, even with short labeling times, no untranslocated AEP precursor is detected suggesting that translocation is co-translational. If the scrl-1 mutation affected signal peptide recognition then one would expect untranslocated AEP precursors to accumulate, but instead we found that this mutation had a preferential effect on biosynthesis of AEP. One possibility is that the scrl-1 mutation does not affect the signal recognition and translational arrest functions of SRP but that translational arrest is maintained.

In higher eukaryotes, translocation is generally thought to be SRP mediated and co-translational (74). In vitro SRP-independent posttranslational translocation across dog pancreas microsomal membranes has recently been demonstrated (61). In S. cerevisiae, there are several proteins for which posttranslational translocation has been demonstrated in vivo (2, 10, 20, 38, 41, 57, 67, 70) or in vitro (27, 28, 55, 70, 75). We show that a mutation in the pro-region of AEP results in its efficient (but slow) posttranslational translocation. Therefore, it appears that AEP can use both posttranslational and co-translational (with SRP at least involved in AEP biosynthesis) translocation pathways.

Materials and Methods

Materials

ProtoSOL and Econofluor were obtained from DuPont, New England Nuclear Research Products (Boston, MA). l-[4,5-3H]leucine and l-[15N]methionine (>800 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). PMSF and 4-hydroxymercuribenzoic acid were from Daiichi Chemical Corp. (San Diego, CA). Casin (Hammersten) was purchased from ICN Pharmaceuticals Inc. (Irvine, CA), and polypropylene glycol (2,000 molecular weight) was from Aldrich Chemical Co., Inc. (Milwaukee, WI). Benzamidine, leupeptin, DMSO, concanavalin A-Sepharose 4B, methyl-α-D-mannopyranoside, and Triton X-100 were from Sigma Chemical Co. (St. Louis, MO). EDTA was from Fisher Scientific, (Pittsburgh, PA). Restriction enzymes, other DNA modifying enzymes, the Random-primed DNA Labeling Kit, and proteinase K were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN), New England Biolabs (Beverly, MA) and Amersham (Arlington Heights, IL). Protein A-Sepharose 4 Fast Flow was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). AEP antiserum was prepared as described previously (40). The drug 5-fluoroorotic acid (5-FOA) was obtained from SCM (Specialty Chemicals, Gainesville, FL).

Growth Media

Complete medium was YEPD, and minimal medium was YNB-glucose (46). Cultures were maintained on YM (40). YM was YM supplemented with uracil (33 mg/ml), leucine (150 mg/ml), and hypoxanthine (100 mg/ml). Cultures were grown in YPDC (1% yeast extract, 1% bactopeptone [Difco Laboratories Inc., Detroit, MI], 1% dextrose, 50 mM sodium citrate, pH 4.0) for transformation (79). For Lys+ strains, lysine, or glutamic acid at a final concentration of 0.1% was used instead of ammonium sulfate in minimal medium since Lys+ strains do not grow on ammonium sulfate as the nitrogen source. GPP* medium (40) was used for the growth of cultures for labeling experiments; it contained 1% glucose, 0.24% Proteose peptone (Difco Laboratories Inc.), 0.17% yeast nitrogen base without amino acids or ammonium sulfate (Difco Laboratories Inc.), 40 mM phosphate buffer pH 6.8, and 0.003% adencine. GC medium is GPP* with 0.4 gm of casein per liter substituted for Proteose peptone. The 5-FOA selective medium was prepared as described by Boeke et al. (11) except the pH was adjusted to 70.

Plasmids and Strains

For DNA manipulations E. coli strains HBlO1 or JM105 were used (59). The E. coli strains used for site-directed mutagenesis were MV1190 and C1236 (24).

The Y. lipolytica haploid needed for the plasmid shuffle was constructed by first mating DX606-3D scr::ADE1 ura31 eu2 pro1 with 21501-4 lys5 leu2 ade1 xpr2 (provided by C. Gaillardin, INRA, Thiverval-Grignon, France) using standard protocols (6, 44). One of the segregants from this cross DX624-3 scr::ADE1 leu2 lys5 ura3 xpr2 was mated with DX388-1B ade1 leu2 trpl ural3. The diploid was sporulated and random spore analysis was done. Three segregants with the desired phenotype of scr::ADE1 leu2 lys5 ura3 were analyzed by Southern blotting to confirm the presence of the scr::ADE1 disruption. One of the segregants DX624-3 scr::ADE1 leu2 lys5 ura3 was transformed with pMR59 which contained the SCRI and ura3 genes and Asia8 an autonomously replicating sequence (provided by P. Fournier, INRA, Thiverval-Grignon, France). Ura+ transformants were selected. After confirming that one of the Ura+ transformants contained the plasmid, the SCR2 chromosomal locus was disrupted with a scr::lyS5 allele (58). The SCR2 disruption was constructed by digesting plNA400 (provided by C. Gaillardin) with Sall to remove the ura3 gene. The plasmid plNA400 is a pBluescript plasmid containing the 3.2-kb HindIII-Xhol SCR2 fragment from which a 570-bp DNA-EcoRV fragment, which contains the entire coding region of SCR2, had been deleted and into which the ura3 gene had been inserted to construct the scr::ura3 disruption (31). The Sall digested plNA400 was treated with Klenow fragment to create blunt ends and with calf intestinal alkaline phosphatase to prevent self-ligation and ligated to the 4.4-kb SphI-BglII lyS5 fragment which had been blunt-ended using T4 DNA polymerase. The resulting recombinant plasmid plMR54 was digested with Apal and NotI to target integration to the SCR2 locus (58). The DX642-3 strain containing plMR59 was transformed and lys+ transformants were selected. The presence of the scr2::lyS5 disruption was confirmed by Southern analysis. This strain was used for the isolation of conditional mutations of SCRI and provided the isogenic background used for the in vivo analysis of the effects of scr1-1 on protein secretion.

DY63 and DY66 were obtained by replacing plMR59 in this strain with plMR63 (SCRI) and plMR66 (SCRI), respectively. For the construction of plMR63, plNA240 which contains the Asia8 and LEU2 fragments was digested with NdeI, blunt-ended, and religated to destroy the NdeI site to create plMR62. A 2.5-kb BamHI/Apal fragment containing SCR1 was isolated, and BamHI linkers were added. The 2.5-kb BamHI SCR1 fragment was subcloned into plMR62 to get plMR63. To obtain plMR66, a 560-bp NdeI/MluI scr1-1 fragment was subcloned into plMR63. For plMR59, the LEU2 fragment in plNA240 was replaced with the 1.7-kb URA3 SacI fragment which gives plMR53, and the 2.5-kb SCR1 BamHI piece described above was subcloned into plMR53.

The glycosylation and NH2-terminal sequencing studies of the P17M mutation were done in a strain derived from CX161-1B ade1 A (40). Details of the plasmid constructions for the deletions of URA3 (400-bp EcoRV fragment) and XPR2 (150-bp Apal fragment) will be described elsewhere. These
deletions were incorporated sequentially into CX161-1B by a two-step gene replacement technique using 5-FOA selection (11). The P17M mutation was incorporated into the ade1 URA3 XPR2 strain by transforming with plasmid pMR118 cut at MluI in the XPR2 promoter region, selecting Ura+ transformants, and then selecting Ura+ Xpr+ strains after growth on 5-FOA. Replacement of the XPR2 deletion was confirmed by Southern analysis. The plasmid pMR118 containing the P17M mutation was constructed by replacing a 1,655-bp SpH1/Xbal fragment in pMR101 with the SpH1/Xbal fragment containing the mutation; pMR110 contains a 2.76-kb SpH1/EcoRI XPR2 fragment and a 1.7-kb SacI URA3 fragment in pBR322.

The P17M mutation was incorporated into DY63 and DY66 in two steps. Since these strains were Xpr+, first the 150-bp ApaI fragment deletion in pIMR118 cut at MluI in the XPR2 promoter region, selecting Ura+ transreplacement technique using 5-FOA selection (11). The P17M mutation deletions were incorporated sequentially into CX161-1B by a two-step gene replacement using 5-FOA (11) to obtain an Xpr- strain. Then the P17M mutation, which yields an Xpr+ phenotype, was incorporated as described above for the ade1 xpr2 ura3 strain.

In Vitro Mutagenesis

Site-directed mutagenesis was done using the Bio-Rad Muta-gene M13 in vitro mutagenesis kit and its detailed protocols and strains; the kit is based on methods described by Kunzel et al. (35). For the two base pair in vitro mutagenesis kit and its detailed protocols and strains; the kit is based on a growing interest in the possibility of using these methods to incorporate mutations into genes of interest. The P17M mutation was incorporated into DY63 and DY66 in two steps. Since these strains were Xpr+, first the 150-bp ApaI fragment deletion in XPR2 was incorporated by the two-step gene replacement using 5-FOA (11) to obtain an Xpr- strain. Then the P17M mutation, which yields an Xpr+ phenotype, was incorporated as described above for the ade1 xpr2 ura3 strain.

Pulse-Chase Immunoprecipitation

Labeling, preparation of cell extracts with glass beads, and immunoprecipitation were done as described previously (40). Cells were grown in GPP medium and resuspended in GC medium at a cell density of 1,000 Klett U (2 × 10^7 cells/ml). For experiments in which cells were shifted to 33°C for one or two hours, GPP cultures were shifted to 33°C for 20 min or 1 h and 20 min, respectively. After harvesting, the cells were resuspended in prewarmed GC and incubated at 33°C for 40 min. After 40 min in GC, 250 ml of l-[4,5-3H]leucine (120-190 Ci/mmol) was added for every 6 ml of cells. Cells were labeled for 45 s, and chased with 3,000-fold excess of cold l-leucine. Cell extracts were prepared and immunoprecipitated with supernatant proteins TCA precipitated as described previously (40) with the following modifications: (a) clarified cell extracts were adjusted to 4% Triton X-100 before the addition of antisera; (b) 50 µl of washed, packed protein A-Sepharose 4 Fast Flow was added to the antiserum and cell extract mixture; and (c) TCA-precipitated supernatant samples were neutralized with 1 M Tris instead of NaOH. Total incorporation of label was measured at selected post-chase time points by scintillation counting of boiled TCA precipitates from two 100 µl samples of labeled cell suspension (40).

Endoglycosidase H Digestion

Endoglycosidase H (endo H) from ICN (Irvine, CA) according to the manufacturer's instructions. Probes were prepared by random primed DNA labeling.

DNA Sequencing

Single (68) and double stranded (15) DNA sequencing was done by the dyeoxerox method (60) using the Sequenase kit (United States Biochemical, Cleveland, OH) and [35S]ATP (9).

Radiosequencing

Radiolabeled cell extracts were prepared as described previously with the following modifications: cells were kept at 23°C during growth and after transfer to GC medium. After 30 min in GC medium, 20 ml of cells were double labeled for 100 s with 1 mCi of l-[35S]methionine and 1 mCi of l-[4,5-3H]leucine and chased with 3,000-fold excess of unlabeled methionine and leucine. Samples were taken as soon as possible and 10 min after the addition of the chase for the 53- and the 55-kD AEP precursor samples, respectively. The immunoprecipitates were run on a 10-15% SDS-PAGE gel for which the precautions recommended by Hunkapiller et al. (33) were taken. The labeled bands were electrophoretically transferred to Immobilon PVDF membrane (Millipore, Bedford, MA) and immunoblotted by double-channel counting with a scintillation counter (model 7500; Beckman Instruments, Inc., Paio Alto, CA).

Protease Protection

The protease protection experiments were based on protocols published for S. cerevisiae (56) and S. lipolytica (21). Cell labeling and the chase were done as in a standard pulse-chase immunoprecipitation experiment except that labeling was for 60 s. A 12-ml sample was taken 1.5 min after addition of the chase and added to 4.6 g of crushed ice containing sufficient sodium azide, leucine, and PMSF to give final concentrations of 10, 2.5, and 2 mM, respectively. Cells were incubated for 15 min at 30°C in sodium thioglycolate (30 mg/ml), 50 mM Tris-HCl (pH 9.1), and 0.8 M KCl. Cells were then incubated for 25 min at 30°C in JD buffer (1 M KCl, 20 mM MES, pH 6.0) containing 4 mg/ml of a lytic enzyme (obtained from J. DeZeeuw, Pfizer, Inc., Groton, CT) prepared from Trichoderma harzianum and ~0.2 mg/ml lyticase (No. LS263; Sigma Chemical Co.). Over 90% of the cells were spheroplasts after this incubation. The cells were washed three times with JD buffer and resuspended in 2 ml of homogenization buffer (0.3 M mannitol, 100 mM KCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.5) (56) containing an inhibitor cocktail to give final concentrations of 2 mM EDTA, 2 mM PMSF, 1 mM benzamidine, 1 µg/ml leupeptin, and 50 µM 4-hydroxymercaptobenzoic acid. Spheroplasts were lysed with 10 strokes by hand in a 10 ml Potter-Elvejhem tissue grinder (No. 358039; Wheaton Scientific, Millville, New Jersey). Gel slices were rehydrated in 50 µl of water and solubilized by shaking overnight at 37°C in 10 ml of ECONOFUOR-Protium mixture (95:5) in glass vials (40).

Glycoprotein Precipitation with ConA

Cells were grown and labeled as described previously except the incubation period was extended to two minutes. Samples were processed as described previously. Of the 600 ml of clarified extract, 385 µl was immunoprecipitated with AEP antisera. 15 µl of the cell extract was diluted with 135 µl of ConA reaction buffer (0.5 M NaCl, 20 mM Tris, pH 7.4, 2% Triton X-100) (19), and 25 µl of packed Con A-Sepharose 4B was added followed by incubation at 4°C for 3 h on a rocking platform. Con A-Sepharose beads were sedimented by centrifugation. The bound supernatant was removed and saved. The beads were washed three times with 1 ml of Con A reaction buffer. Bound glycoproteins were eluted with 200 µl of 300 mM methyl-α-d-mannopyranoside, 2 mM PMSF, 0.5 M NaCl, 20 mM Tris, pH 7.4, 2% Triton X-100 at room temperature for 1 h with shaking. After the beads were pelleted, the supernatant containing eluted glycoproteins was removed. The ratio of bound counts to total counts was determined by scintillation counting of the bound and unbound fractions.

RNA Analysis

Total RNA, prepared as described by Davidow et al. (18), was electrophoresed on an agarose gel containing formaldehyde (59). The RNA was transferred to Zeta probe nylon membrane (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. Probes were prepared by random primed DNA labeling.

PAGE and Fluorography

Proteins were analyzed by electrophoresis in 10 to 15% linear gradient polyacrylamide gels (40). For immunoprecipitations, 25 µl of sample, equivalent to 0.375 ml of suspended cells, was applied per lane. Extracellular proteins precipitated by TCA were dissolved in 250 µl of Laemmli buffer, and 25 µl, equivalent to 0.3 ml of suspended cells, was loaded per lane. After electrophoresis, gels were stained with Coomassie brilliant blue and destained in 10% methanol, 15% acetic acid. The gels were treated with DMSO and the scintillator 2,5-diphenyloxazole. The treated gels were dried and exposed to preflashed Kodak X-Omat AR film and stored at -80°C.
and centrifuged for 5 minutes at 900 g. 6 ml of supernatant liquid was divided into four equal portions. In some samples, Triton X-100 and/or protease K were added to final concentrations of 0.4% and 0.5 mg/ml, respectively. The samples were incubated for 1 h at 0°C, TCA added to 10% (vol/vol), and after several hours incubation on ice the precipitate was washed with cold acetone and dried in the Speed Vac (Savant Instruments, Inc., Farmingdale, NY). To each sample, 200 µl of 50 mM Tris-HCl (pH 6.8), 1 mM MgCl2, 1 mM CaCl2, 1 mM leucine was added. The solutions were made up to 1% SDS and 2 mM PMSF, vortexed, and incubated at room temperature for 10 min. Then Triton X-100 was added to 4% final concentration, the antibody added, and the standard immunoprecipitation protocol followed (40).

Results

Isolation of Conditional Alleles of SCR1

To begin elucidating the in vivo functions of the SRP 7S RNA, we isolated a conditional mutation in SCR1 by site-directed mutagenesis. The mutation was designed based on the consensus sequence -GNAR- present in the stem-loop of domain III of all sequenced 7SL RNAs (14). The mutant allele scr1-l contained two base changes (G to A at 129 and A to T at 131) (Fig. 1), and it was shown to result in temperature-sensitive growth using a plasmid shuffle (11). A haploid in which both SCR1 and SCR2 chromosomal loci were disrupted and in which the SCR1 and URA3 genes were carried on an autonomously replicating plasmid (pIMR59) was transformed with an autonomously replicating plasmid carrying scr1-l and LEU2 (pIMR66). Leu+ transformants were selected, and plated on medium containing 5-FOA which inhibits growth of URA3+ cells. A strain [DY66 (scr1-l)] carrying scr1-l on the LEU2 plasmid grew on 5-FOA at 14 and 23°C but not at 33°C. The scr1-l plasmid was rescued from the strain and rescreened in the plasmid shuffle which confirmed that the temperature-sensitive phenotype was linked to the plasmid. The 560-bp NdeII/MluI fragment of the rescued plasmid was sequenced to confirm that it contained the scr1-l allele.

The scr1-l Allele at the Normal Chromosomal Locus Cannot Support Growth

We attempted to replace SCR1 with the scr1-l allele at its normal chromosomal locus to construct a strain for in vivo studies of the effects of scr1-l on protein secretion. Integrating plasmids containing the URA3 gene and either the SCR1 or scr1-l allele were digested to target integration to the scr1::ADE1 locus of DY63 [ASCRI, ASCR2, pIMR63 (LEU2, SCR1)]. Ura+ transformants were selected and grown in YEPD medium supplemented with leucine to allow for loss of pIMR63. In the Leu+ derivatives obtained, the only copy of the 7S RNA gene present should be that integrated at scr1::ADE1. These Leu+ strains were then grown on 5-FOA to select for a homologous recombination event which should leave either the original scr1::ADE1 allele or the recently integrated SCR1 or scr1-l alleles at the normal chromosomal locus.

For the SCR1 integrant, a successful transplacement occurred which was confirmed by Southern analysis (data not shown). However, the scr1-l integrants were unable to lose the SCR1 LEU2 plasmid (pIMR63) even after growth in YEPD supplemented with leucine at 23°C for ~48 generations. No loss of pIMR63 was seen among the more than 3,200 colonies scored in contrast to the >50% loss frequency seen for the SCR1 integrants. Loss of pIMR63 in the scr1-l integrants was also tested at 18°C, and no Leu+ colonies were found among the >4,000 scored indicating that the chromosomal scr1-l could not support growth even at this lower temperature. Perhaps, higher levels of scr1-l 7S RNA are
produced from an autonomously replicating plasmid (copy number of 2 to 3) (23) than from its chromosomal locus, and at 23°C these higher levels can compensate for the defect caused by the structural alteration of the scrI-75S RNA.

We next investigated how quickly cells were irreversibly inactivated during incubation at 33°C so that they could no longer recover and grow when transferred back to 23°C (Fig. 2). DY63 (SCRI) and DY66 (scrI-1) were grown at 23°C and split at time zero; half was shifted rapidly to 33°C, and the other half was kept at 23°C. Samples were taken and plated on YM+ at 23°C. At least half the number of DY66 (scrI-1) cells originally present could recover and grow even after 6 h at the nonpermissive temperature.

**The scrI-1 Allele Preferentially Affects the Levels of a Secretory Protein**

The isogenic strains DY63 (SCRI) and DY66 (scrI-1) were examined in pulse-chase immunoprecipitation experiments to obtain in vivo evidence for SRP involvement in protein secretion. AEP, which can account for up to 2% of total cell protein (40, 43), was used as the reporter molecule. AEP posttranslational processing results in several intracellular precursors (40). The earliest and largest precursor detected in wild type cells is a 55-kD polypeptide which presumably has been translocated into the ER because it contains 2 kD of N-linked carbohydrate (40) and it lacks the signal peptide (21, unpublished data). Even with pulse-labeling times as short as 30 s, untranslated AEP precursors are not found (data not shown), suggesting that translocation is co-translational.

Our original expectation was that an untranslated AEP precursor might accumulate in DY66 (scrI-1) but not in DY63 (SCRI). This precursor should still retain the 15 amino acid signal peptide but it should lack the 2 kD of N-linked carbohydrate, thus it would be predicted to have a mobility on SDS-PAGE similar to that of the 55-kD precursor. For both DY63 (SCRI) and DY66 (scrI-1), the only precursors detected at early time points after the cells were shifted to 33°C after 1 h were about 55 kD. Treatment with endo H revealed that both precursors contained about 2 kD of N-linked carbohydrate strongly suggesting that they had been translocated across the ER membrane (Fig. 3). Thus, we obtained no evidence for an untranslated AEP precursor in scrI-1 cells, and data presented below suggests that we would have been able to detect such a precursor.

We did notice, however, that in DY66 (scrI-1) the amount of labeled 55-kD precursor immunoprecipitated at all timepoints examined was consistently reduced in comparison to the levels detected in DY63 (SCRI) (Fig. 4, a and c). These differences were much greater than the 12% decrease (average of five experiments) in total protein synthesis found for DY66 (scrI-1) versus DY63 (SCRI). To quantitate the amounts of the 55-kD precursor immunoprecipitated, bands containing the precursor were cut from the gel, rehydrated, and counted in a scintillation counter. The absolute values of counts in 55-kD AEP precursor/total counts incorporated × 100% varied considerably from experiment to experiment; however, the relative ratios of the value for DY66 (scrI-1) divided by that for DY63 (SCRI) were fairly consistent within an experiment. For the 0, 1.5, and 3 min time points the ratios were 0.37 ± 0.22 (n = 5), 0.32 ± 0.18 (n = 4), and 0.28 ± 0.10 (n = 5) (mean ± SD, n = number of determinations).

Therefore, ~68% reduction in the specific rate of AEP 55-kD precursor synthesis was observed with the scrI-1 mutation. As would be predicted based on the above results, the levels of mature AEP secreted from DY66 (scrI-1) were also lower than from DY63 (SCRI) (Fig. 4 b), although the decrease was not as great as for the intracellular 55-kD AEP precursor (see Discussion).

After a 2 h shift to 33°C, a similar result of an approximate 75% reduction in AEP precursor levels in DY66 was also obtained. However, after a 15 min shift to 33°C, little or no effect on AEP biosynthesis was observed.

We examined the effects of the scrI-1 mutation on other secreted proteins by measuring Con A bindable counts after either a 0, 1, or 2 h shift to 33°C. Con A binds mannose residues which are added in the secretory pathway (45) and to some extent in the cytoplasm and nucleus (29). Cells were labeled for 2 min followed by a chase. Samples were taken immediately, and intracellular extracts were incubated with Con A-Sepharose 4B. Total bound and unbound counts were measured, and the ratios of total bound counts to total counts (bound plus unbound) were calculated (Table I). After a 1 or 2 h shift to 33°C, total counts in glycoproteins were reduced on average by 27% in DY66 (scrI-1) as compared to DY63 (SCRI); whereas, when cells were grown and labeled at 23°C, the counts in the glycoproteins were comparable. The reduction of 27% in Con A bindable counts in the mutant strain is much less than the 68% reduction of the levels of AEP 55-kD precursor suggesting that the effect on most other glycoproteins traversing the secretory pathway is not as severe as for AEP.

To rule out two possible explanations for the lower levels of AEP precursors present in DY66 (scrI-1), SCRI RNA levels and AEP mRNA levels were compared in DY63 (SCRI) and DY66 (scrI-1) 1 h after a shift to 33°C. Total RNA from both strains was isolated after the temperature shift. Northern blots were probed with labeled SCR1 and XPR2 (AEP) gene fragments and with a probe for rRNA as a control for RNA recovery and loading. The levels of both AEP mRNA and 7S RNA present in the DY66 (scrI-1) were comparable to those seen in DY63 (SCRI) (data not shown). Therefore, lower levels of AEP mRNA or 7S RNA cannot account for the decrease in AEP precursors detected in the mutant scrI-1 cells.

| scrI-1 | SCRI |
|-------|-----|
| Endo H | + | + |
| - | + | / |
| - | - | + |

Figure 3. The 55-kD AEP precursors found in DY63 (SCRI) and DY66 (scrI-1) after 1 h at 33°C contain ~2 kD of N-linked carbohydrate. Immunoprecipitates from 0 time samples were treated with endo H and electrophoresed on a 10 to 15% linear gradient SDS-PAGE gel. The figure includes two sections of the same gel. For the three lanes on the left, the film was exposed for 75 d and for the two lanes on the right, 12 d. The lanes contain, from left to right, DY66 (scrI-1) immunoprecipitate, endo H digested DY66 (scrI-1) immunoprecipitate, endo H-digested and -undigested DY66 (scrI-1) immunoprecipitates combined, DY63 (SCRI) immunoprecipitate, and endo H-digested DY63 (SCRI) immunoprecipitate.
Detection of an Untranslocated AEP Precursor Suggests That scr1-1 Affects Synthesis and Not Translocation of AEP: A Mutated AEP Can Be Translocated Posttranslationally

The decreased level of the 55-kD AEP precursor in DY66 (scr1-1) after a shift to 33°C could result from a decrease in AEP synthesis and/or from synthesis of an untranslocated AEP precursor which is short lived or extremely sensitive to endogenous proteases and therefore undetectable even with a very short pulse. The second possibility was largely eliminated by the fortuitous discovery of a mutated AEP (P17M) which is translocated posttranslationally. In P17M the proline in position 17, the second position in the 142 amino acid pro-peptide, was changed to methionine (see top of Fig. 7). The P17M allele of AEP was substituted for the wild type gene at its normal chromosomal locus, and the P17M strain was examined by pulse-chase immunoprecipitation experiments at either 23°C or after a 1-h shift to 33°C. The earliest detected AEP precursor was ~53 kD (Fig. 5a), and the 53 kD precursor was completely chased to a 55-kD precursor (Fig. 5b). Endo H digestions of the 53- and 55-kD precursors demonstrated that the 53-kD form is unglycosylated, and the 55-kD form contains 2 kD of N-linked carbohydrate (Fig. 6).

The endo H results suggest that the 53-kD AEP precursor...
Table I. Total Glycoprotein Precipitated by Concanavalin A

| Strain                  | Time at 33°C (h) | Total incorporation DY66/DY63 × 100% | CPM         | Bound | Bound/Total* | (Bound/Total for DY66)/ (Bound/Total for DY63) |
|------------------------|-----------------|-------------------------------------|-------------|-------|-------------|---------------------------------------------|
| DY66 (scr1-1)          | 0               | 106                                 | 1,277,000   | 26,800| 0.031       | 103%                                        |
| DY63 (SCRI)            | 0               | 1,616,000                           | 32,600      | 0.030 |             |                                             |
| DY66 (scr1-1)          | 1               | 97                                  | 1,087,000   | 20,400| 0.027       | 77%                                         |
| DY63 (SCRI)            | 1               | 1,371,000                           | 32,400      | 0.035 |             |                                             |
| DY66 (scr1-1)          | 1               | 99                                  | 1,060,000   | 23,200| 0.021       | 77%                                         |
| DY63 (SCRI)            | 1               | 808,500                             | 23,100      | 0.028 |             |                                             |
| DY66 (scr1-1)          | 2               | 95                                  | 1,472,000   | 17,400| 0.012       | 67%                                         |
| DY63 (SCRI)            | 2               | 1,275,000                           | 22,900      | 0.018 |             |                                             |
| DY66 (scr1-1)          | 2               | 119                                 | 731,000     | 38,200| 0.050       | 70%                                         |
| DY63 (SCRI)            | 2               | 702,500                             | 52,300      | 0.069 |             |                                             |

* CPM bound to ConA divided by CPM bound plus CPM unbound.

is not translocated in which case it should still contain the signal peptide. Therefore, NH₂-terminal radiosequencing of the 53- and 55-kD AEP precursors was done. Cells were grown at 23°C and double labeled with [³⁵S]methionine.
through the same temperature regime used for spheroplast elimination. To test this possibility, cells were pulse-labeled and taken during sample preparation despite addition of sodium azide. Precursors that were labeled posttranslationally were most likely located outside P17M AEP 53-kD precursor is most likely located outside P17M AEP 53-kD precursor is most likely located outside the cell extract. The sequence at the top of the figure is the first 20 NH2-terminal amino acids of the prepro-region of AEP containing the P17M mutation; // indicates the signal peptide cleavage site.

Met Lys Leu Ala Thr Ala Phe Thr Ile Leu10 Thr Ala Val Leu Ala // Ala Met Leu Ala Ala20

\[
\text{Met Lys Leu Ala Thr Ala Phe Thr Ile Leu10 Thr Ala Val Leu Ala // Ala Met Leu Ala Ala20}
\]

[3H]leucine. After the chase, samples were taken immediately and 10 min after addition of the chase for the 53- and 55-kD precursors, respectively. Radiosequencing of the 53-kD precursor yielded methionine and leucine residues in cycles 1 and 3, respectively (Fig. 7a). This arrangement indicates that the 53-kD precursor begins with the initiator methionine and that it must contain the signal peptide. For the 55-kD precursor, a methionine in cycle 2 and a leucine in cycle 3 were detected indicating that the 55-kD form begins after the signal peptide cleavage site (Fig. 7b). The glycosylation and radiosequencing results both strongly suggest that the 53-kD precursor is not translocated and that the 55-kD precursor is translocated. Thus the P17M AEP molecule appears to be posttranslationally translocated. The fact that the P17M 53-kD untranslocated precursor was readily detected strongly suggests that a wild type untranslocated AEP precursor would also be detectable at 33°C in DY66 (scl-1).

To demonstrate that the P17M 53-kD AEP precursor is untranslocated while the P17M 55-kD AEP precursor is translocated, we performed protease-protection experiments. We labeled intact cells, made spheroplasts, lysed the spheroplasts, and did protease-protective experiments on the lysate. We could demonstrate that both the P17M and wild type 55-kD precursors were protected from proteinase K digestion in the absence of Triton X-100 but not in its presence (data not shown). However, we could not detect the P17M 53-kD AEP precursor even in the control where neither proteinase K nor Triton X-100 was added which suggests that the P17M 53-kD AEP precursor is proteolytically degraded during preparation of cell extracts from spheroplasts.

One explanation for the absence of the P17M 53-kD precursor is that it was being posttranslationally translocated during sample preparation despite addition of sodium azide. In this case, only the 55-kD precursor would be detected. To eliminate this possibility, cells were pulse-labeled and taken through the same temperature regime used for spheroplasting and cell lysis. A cell extract was prepared by homogenization with glass beads which should break open both cells and intracellular compartments. Both the P17M 53- and 55-kD AEP precursors were detected, but neither precursor was protected from proteinase K even in the absence of Triton X-100 (data not shown). Therefore, the 53-kD precursor does not appear to be posttranslationally translocated during sample preparation, and this is consistent with in vitro results showing that energy is required for posttranslational translocation (28, 55, 75). We conclude that the P17M 55-kD precursor is in a membrane-bound compartment and that the P17M AEP 53-kD precursor is most likely located outside a membrane-protected compartment where it is more accessible to proteases present in the cell extract.

Detection of the P17M 53-kD untranslocated precursor demonstrates that an untranslocated precursor should be stable enough to be detected in DY66 (scl-1) or wild type. Taken together with the decrease in the levels of the 55-kD AEP precursor in DY66 (scl-1), these results strongly suggest that the scl-1 mutation preferentially affects AEP synthesis and not translocation. Based on the models of SRP function, we believe the decrease in AEP synthesis may be due to maintenance of translational arrest (see Discussion).

**The scl-1 Allele Does Not Affect the Levels of P17M AEP Precursors**

Results obtained with P17M suggest that full length AEP precursors are being synthesized in the cytoplasm and posttranslationally translocated into the ER. Results from wild type suggest that translocation is co-translational and results with the scl-1 mutation suggest that SRP is at least involved in AEP synthesis. The posttranslational translocation of P17M suggests that signal peptide recognition by SRP does not occur, whereas, it seems likely that for the scl-1 mutation signal peptide recognition does occur. Therefore, it would be predicted that when the P17M and scl-1 mutations were combined in the same strain, the mutated AEP should
Table II. Synthesis after 1 h at 33°C of P17M AEP Precursors in Cells Containing scr1-1 or SCR1

| Time after chase (min) | Strain               | Number of experiments | (Counts in AEP precursors/Total counts incorporated) x 100%* Mean (SD) |
|------------------------|----------------------|-----------------------|---------------------------------------------------------------|
|                        |                      |                       | 53 kD                                | 55 kD                                | 53 kD + 55 kD |
| 0                      | DY66-P17M (scr1-1)   | 3                     | 0.206 (0.036) | 0.124 (0.035) | 0.330 (0.059) |
| 0                      | DY63-P17M (SCR1)     | 2                     | 0.193 (0.013) | 0.108 (0.020) | 0.301 (0.033) |
| 1                      | DY66-P17M (scr1-1)   | 2                     | 0.201 (0.033) | 0.204 (0.012) | 0.439 (0.081) |
| 1                      | DY63-P17M (SCR1)     | 1                     | 0.195       | 0.244       | 0.439         |
| 1.5                    | DY66-P17M (scr1-1)   | 3                     | 0.158 (0.067) | 0.284 (0.199) | 0.456 (0.257) |
| 1.5                    | DY63-P17M (SCR1)     | 2                     | 0.165 (0.009) | 0.256 (0.061) | 0.421 (0.070) |

* Cells were grown at 23°C, transferred to 33°C for 1 h, labeled for 45 s with [3H] leucine, and a standard immunoprecipitation was done. Duplicate SDS-PAGE gels were run for twice the normal length of time to increase the separation of the 53- and 55-kD AEP precursors. Bands were detected by fluorography, cut out, and counted.

Discussion

Translational Arrest?

We have isolated a temperature sensitive mutation in the SCR1 gene coding for one of the two functional SRP 7S RNAs synthesized in Y. lipolytica. The mutation scr1-1 changes both conserved bases in the conserved sequence -GNAR- in the loop of the domain III stem loop (14). It is not known if either of the base changes alone would cause the phenotype. The scr1-1 allele could not support growth when integrated at its normal chromosomal location. The plasmid copy number is estimated to be two to three (23) and the increased copy number, perhaps combined with a different transcription rate on the plasmid, probably results in high enough levels of scr1-1 7S RNA to compensate for the functional defect(s).

Whether scr1-1 causes defects in SRP assembly or loss of function of existing SRP molecules remains to be determined. No effect on AEP synthesis was observed at 23°C or at 15 min after the shift to 33°C, while the effects after 1 or 2 h were similar. This implies that existing SRP particles did not rapidly lose functionality. On the other hand 1 h is less than half the doubling time, and if the turnover rate of SRP 7S RNA is low, then defective SRP assembly is a less likely explanation for the effect on AEP synthesis. The fact that the loss of cell viability was not much more rapid than the decrease in AEP synthesis after a shift to 33°C is consistent with the primary defect of the scr1-1 mutation being the decrease in AEP synthesis (and presumably synthesis of other SRP "dependent" proteins).

Nearly 70% of tetraloops (four nucleotide loops) in rRNAs are either -UNCG- or -GNRA- (25, 77). These two tetratemplate sequences also occur in many other RNAs like 7S RNA, the catalytic RNA of RNase P and the self-splicing RNAs (71). The -GNAR- consensus in the stem loop of domain III of the 7S RNA (and specifically the GAUA in Y. lipolytica) fits the -GNRA- tetratemplate consensus. In addition, the stem loop domain IV of the S. pombe 7S RNA, shown to be important for function (37), also fits the -GNRA- tetratemplate consensus. The solution structures of RNA hairpins containing a -GCAA- or -GAAA- tetraloops have been reported recently (32). The hairpin is very compact in shape and has many intramolecular interactions which contribute to its unusual stability (32) and which could provide nucleation sites for correct folding of RNAs (16). The -AUUA sequence in the scr1-1 mutation contains two changes from the -GNRA- consensus. It is likely that it affects folding or stability of the 7S RNA, but the possibility of sequence-specific interactions with SRP polypeptides or even the SRP receptor protein cannot be excluded.

A major result of this study is that a mutation in the 7S RNA component of SRP preferentially affects the synthesis of AEP, a secreted protein from Y. lipolytica. Although the results with scr1-1 do not support a role in protein translocation, they constitute in vivo evidence for involvement of SRP with a secreted gene product. The three functions of SRP as defined in vitro are signal recognition, translational arrest, and translocation promotion. A possible explanation of the data which fits current models is that signal recognition and translational arrest were not affected by the scr1-1 mutation, but that it resulted in decreased ability to release translocation arrest after interaction with the SRP receptor or in decreased efficiency of the interaction of the nascent chain/ribosome/SRP complex with the SRP receptor. Since SRP and translational arrest are released after the complex binds to the SRP receptor, this less efficient interaction would lead to the maintenance of translational arrest of AEP synthesis. Translational arrest by SRP has been demonstrated in several in vitro systems (see reference 78), but the only other in vivo evidence for translational arrest is quite indirect (76). We believe that the effect of scr1-1 mutation on AEP synthesis is
the most direct indication that translational arrest is occurring in vivo. Whether or not it is an essential in vivo function still remains to be determined.

In vitro studies in higher eukaryotes suggested that the loops in domains III and IV of the SRP 7S RNA are bound by Srp19p (64), and that this binding is required for Srp54p binding (64, 65). However, recent results suggest that domain III may interfere with Srp54p binding in the absence of Srp19p and that after Srp19p binding there is a conformational change in the 7S RNA so that Srp54p now can bind to the stem loop in domain IV (54, 80). Since Srp54p interacts with the signal peptide (34), we originally expected scrI-I to decrease the affinity of SRP for the signal peptide resulting in accumulation of an untranslocated AEP precursor. Instead, our results suggest that the mutated loop might affect the conformation or binding of Srp68p, which is believed to interact with the SRP receptor (64-66), or that SRP 7S RNA interacts directly with SRP receptor.

The total counts incorporated and the ratio of Con A bound counts to total counts varied substantially from experiment to experiment (Table I). However, within an experiment the comparison of the ratios of bound/total counts was quite consistent with values for DY66 (scrI-I) being 67-77% of those for DY63 (SCRI) for an average decrease of 27%. This value is somewhat of an underestimate as some labeled glycoproteins are cytoplasmic or nuclear (29). However, unless they are a large percentage of the total glycoproteins, the decrease in synthesis for secreted proteins determined in the Con A experiment is significantly less than the decrease in AEP synthesis. This suggests that biosynthesis of other secretory proteins is not as severely affected by the scrI-I defect as is AEP biosynthesis. This is consistent with translational arrest by SRP not necessarily being complete (78) and with the degree of arrest depending on the specific signal peptide.

The decrease in secreted AEP synthesized in DY66 (scrI-I) compared to DY63 (SCRI) was also about threefold less than for the 55-kD AEP precursor. Calculated based on the percentage of total counts incorporated found in secreted AEP, the decrease averaged 22% for the last three samples in Fig. 4 b. The reason for this difference is unknown. The use of immunoprecipitation to measure levels of the 55-kD AEP precursor, a transient intermediate, may underestimate the decrease in AEP synthesis. If translational arrest results in a range of less than full-length precursors, they might be less efficiently precipitated and they might not be detected because they would be spread out over the gel lane. Early in the chase, more labeled partially completed precursor molecules would be expected in DY66 (scrI-I) than in DY63 (SCRI) because of the maintenance of translational arrest. However, eventually arrest would be released and this previously undetected label would ultimately accumulate in secreted AEP.

Co- and Posttranslational Translocation

Another significant result of this study is the posttranslational translocation of the mutated P17M AEP. Although posttranslational protein translocation has been demonstrated both in vivo and in vitro for procaryotes (41) and S. cerevisiae (2, 10, 20, 27, 28, 38, 42, 55, 57, 67, 70, 75), Y. lipolytica is the most evolutionarily advanced organism in which in vivo posttranslational translocation has been shown to occur. Y. lipolytica is dimorphic, growing as either yeast-like or filamentous forms, and in many ways such as the organization of its rRNA genes (17, 72) it appears more closely related to the filamentous fungi than to other yeast genera. Based on 18S rRNA sequences, the evolutionary distance between Y. lipolytica and S. cerevisiae (5) is comparable or greater than that between S. cerevisiae and S. pombe (W. Weisburg, personal communication). A comparison of snRNAs in various yeasts revealed that the sizes of the Y. lipolytica snRNAs were much closer to those for human than were the snRNAs from S. cerevisiae (53). By several criteria the Y. lipolytica SRP 7S RNA is more higher eucaryotic-like than the S. cerevisiae homologue. The Y. lipolytica SRP 7S RNAs are closer in size and seemingly secondary structure, 270 nucleotides (30, 46) versus 300 for human (74) and 519 for the S. cerevisiae SCR1 gene product (22). In Y. lipolytica the SCR genes are essential for growth (31) and not just important as in S. cerevisiae (22). Finally, higher eucaryotes have multiple transcribed SRP 7S RNAs (81), and Y. lipolytica is the only genetically tractable lower eucaryote having more than one functional SRP 7S RNA.

The conclusion that the P17M AEP is translocated posttranslationally was based on several findings. The kinetics of the pulse-chase experiments fit a precursor-product relationship for the 53- and 55-kD precursors. An untranslocated precursor would be expected to contain the signal peptide and to lack N-linked carbohydrate as was found for the P17M 53-kD AEP precursor. A translocated precursor would be expected to lack the signal peptide and to contain N-linked carbohydrate as was found for the P17M 55-kD AEP precursor. The protease-protection experiments demonstrate that the P17M 55-kD AEP precursor is located in a membrane-bound compartment and are consistent with a cytoplasmic location for the P17M 53-kD AEP precursor.

The results with P17M in a wild-type background and with DY66-P17M suggest that the signal peptide was not recognized by SRP. The P17M mutation may affect the conformation or accessibility of the signal peptide. In vitro experiments with preprolactin suggest that subtle sequence changes beyond the signal cleavage site could affect signal peptide function, and co-translational translocation was less efficient in the preprolactin mutants (4). However, translational arrest by SRP in vitro in the absence of membranes did not seem to be affected (4).

The presence of the 55-kD AEP precursor in the earliest time point samples raised the possibility that some translocation of P17M AEP precursors was occurring co-translationally. If this were the case then the level of 55-kD synthesis in DY66-P17M would be expected to be lower than in DY63-P17M. However, no significant difference was found suggesting that all or nearly all the 55-kD AEP precursor was translocated posttranslationally. If the P17M precursor does not interact with SRP, then how it is targeted to the ER membrane and whether or not it uses the same translocation machinery as used for SRP-mediated co-translational translocation are open questions. It should be noted that the translocation of P17M AEP is kinetically quite inefficient compared to co-translational translocation of wild type AEP.

The results obtained with the mutated P17M AEP are not inconsistent with co-translational translocation (with SRP at least involved in AEP synthesis) being the major pathway for
AEP translocation in wild type cells. In fact, the demonstration that an untranslocated AEP precursor can be detected in the mutant strengthens the conclusion (based on the absence of untranslocated precursors even after labeling times as short as 30 s) that translocation is co-translational in the wild type. Recently in vitro evidence for posttranslational and SRP-mediated co-translational translocation was obtained for dog pancreas microsomal membranes (61), and in vivo evidence for involvement of SRP homologues in protein translocation in S. cerevisiae may be forthcoming (48). However, assuming that SRP is involved not only in synthesis but also in translocation of AEP, then AEP would be the first protein for which there would be in vivo evidence for both posttranslational and SRP-mediated co-translational translocation. The AEP results are consistent with a recent E. coli model where the co-translational pathway is mediated by SRP-like ribonucleoprotein and the posttranslational pathway is a salvage pathway (50).

We thank C. Gaillardin and P. Fournier for ARS18, LV5S, and SCR2, J. Gardner at the University of California at Davis, Protein Structure Laboratory for NH2-terminal sequencing, J. DeZeeuw for the T. harzianum lytic enzyme, and C. Enderlin, C. Gaillardin, and M. Poritz for helpful discussions.

This work was supported by The National Science Foundation and the California Agricultural Experiment Station.

Received for publication 19 June 1991 and in revised form 16 October 1991.

**Note Added in Proof.** Recently, Hann and Walter (1991. Cell. 67:131-144) reported cloning of a NH2-terminal sequence corresponding to the signal recognition particle, J. DeZeeuw for the Trichoderma harzianum lytic enzyme, and C. Enderlin, C. Gaillardin, and M. Poritz for helpful discussions.

**References**

1. Bourret, R. A. L., and P. Walter. 1989. Prepro-carboxypeptidase Y and a truncated form of pre-invertase, but not full-length pre-invertase, can be posttranslationaly transferred across microsomal vesicle membranes from Saccharomyces cerevisiae. J. Cell Biol. 106:1075-1081.

2. Hansen, W., and P. Walter. 1987. In vitro translocation across the yeast endoplasmic reticulum: ATP-dependent posttranslational translocation of the prepro-a-factor. Cell. 35:397-406.

3. Han, E. C., M. A. Poritz, and P. Walter. 1989. Saccharomyces cerevisiae and Schizosaccharomyces pombe contain a homolog to the 54-kD subunit of the signal recognition particle that in S. cerevisiae is essential for growth. J. Cell BioL 109:3223-3230.

4. Hansen, W., and P. Walter. 1988. Prepro-carboxypeptidase Y and a truncated form of pre-invertase, but not full-length pre-invertase, can be posttranslationally transferred across microsomal vesicle membranes from Saccharomyces cerevisiae. J. Cell Biol. 106:1075-1081.

5. Hansen, W., and P. Walter. 1986. In vitro translocation across the yeat endoplasmic reticulum: ATP-dependent posttranslational translocation of the prepro-a-factor. Cell. 35:397-406.

6. Harter, K. W., S. R. Haltiwanger, D. G. Holt, and W. G. Kelly. 1989. Glycosylation in the nucleus and the cytoplasm. Annu. Rev. Biochem. 58:841-874.

7. He, G. J., M. Beckersch, V. Ribes, D. Dieterly, and C. Gaillardin. 1989. Two genes encode 7SL RNAs in the yeast Saccharomyces. J. Bacteriol. 164:347-350.

8. He, F., D. Yaver, J. M. Beckersch, D. Orygdiaski, and C. Gaillardin. 1990. The yeast Yarrowia lipolytica has two functional, signal recognition par- ticle 7SL RNA species. Curr. Genet. 17:289-292.

9. Heus, H. A., and A. Pardi. 1989. Structural features that give rise to the unusual stability of RNA hairpins containing GNR loops. Science (Wash. DC). 253:191-194.

10. Hunkapiller, M. W., E. L. Laij, F. Ostrand, and P. L. E. Hood. 1983. Isola- tion of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. Methods Enzymol. 112:2604-8608.

11. Krieg, U. C., P. Walter, and A. E. Johnson. 1986. Photocrosslinking of the signal sequence of nascent preprolactin to the 5-kilodalton peptide of the signal recognition particle. Proc. Natl. Acad. Sci. USA. 83:8604-8608.

12. Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. 154:367-382.

13. Lafferty, L., P. D. Garcia, R. N. Harkins, L. Cousens, A. Ulrich, and P. Walter. Topology of signal recognition particle receptor in endoplasmic reticulum membrane. Nature (Lond.). 318:334-338.

14. Liou, X., P. Breenwald, and J. A. Wise. 1989. Genetic analysis of Schizo- saccharomyces pombe 7SL RNA: A structural motif that includes a con- served tetranucleotide loop is important for function. Proc. Natl. Acad. Sci. USA. 86:4137-4141.

15. Lohle, S. J., and H. Bushey. 1986. In vivo evidence for posttranslational translocation and signal cleavage of the killer preprotoxin of Saccharo- myces cerevisiae. Mol. Cell Bioll. 6:4274-4280.

16. Matsuda, S., and D. M. Ogrydziak. 1989. A novel novel for dipeptidyl aminopeptidase processing sites in the alkaline extracellular protease of Yarrowia lipolytica. J. Bacteriol. 264:6037-6043.

17. Matsuda, S., J. Fukayama, R. A. Wing, and D. M. Ogrydziak. 1988. Intra- cellular precursors and secretion of the alkaline extracellular protease of Yarrowia lipolytica. Mol. Cell Bioll. 8:4904-4916.

18. McPheeters, M., and S. R. Haltiwanger. 1990. Prokaryotic secretion. Cell. 61:739-741.

19. Ngsee, J., W. Hansen, P. Walter, and M. Smith. 1989. Cassette mutagenic
analysis of the yeast invertase signal peptide: effects on protein transloca-

43. Osawa, T., and T. Tsuij. 1987. Fractionation and structural assessment of oligosaccharides and glycopeptides by use of immobilized lectins. *Ann. Rev. Biochem.* 56:21–42.

44. Poritz, M. A., V. Siegel, W. Hansen, and P. Walter. 1988. Small ribonucleo-

cleoproteins in *Schizosaccharomyces pombe* and *Yarrowia lipolytica* homo-

genous to signal recognition particle. *Proc. Natl. Acad. Sci. USA.* 85: 4315–4319.

45. Osawa, T., and T. Tsuij. 1987. Fractionation and structural assessment of oligosaccharides and glycopeptides by use of immobilized lectins. *Ann. Rev. Biochem.* 56:21–42.

46. Poritz, M. A., V. Siegel, W. Hansen, and P. Walter. 1988. Small ribonucleo-

cleoproteins in *Schizosaccharomyces pombe* and *Yarrowia lipolytica* homo-

genous to signal recognition particle. *Proc. Natl. Acad. Sci. USA.* 85: 4315–4319.

47. Ogrydziak, D. M., and S. J. Scharf. 1982. Alkaline extracellular protease pro-

duced by *Saccharomyces lipolytica* CX161-1B. *J. Gen. Microbiol.* 128:1225–1234.

48. Osawa, T., and T. Tsuij. 1987. Fractionation and structural assessment of oligosaccharides and glycopeptides by use of immobilized lectins. *Ann. Rev. Biochem.* 56:21–42.

49. Rapoport, T. A. 1990. Protein transport across the ER membrane. *Trends Biochem. Sci.* 15:355–358.

50. Rapoport, T. A. 1991. A bacterium catches up. *Nature (Lond.).* 349:107–108.

51. Ribes, V., P. Dehoux, and D. Tollervey. 1988. 7SL RNA from *Schizosac-

charomyces pombe* is encoded by a single copy essential gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:4511–4517.

52. Ribes, V., K. Romisch, A. Giner, B. Dobberstein, and D. Tollervey. 1990. *E. coli* 4.5S RNA is part of a ribonucleoprotein particle that has properties related to signal recognition particle. *Cell.* 63:591–600.

53. Roiha, H., E. O. Shuster, D. A. Brow, and C. Guthrie. 1989. *snRNAs from budding yeasts: phylogenetic comparisons reveal extensive size variation. Gene.* 82:137–144.

54. Rothblatt, J. A., and D. I. Meyer. 1986. Secretion in yeast: reconstitution of the translocation and glycosylation of α-factor and invertease in a homol-
genous cell-free system. *Cell.* 44:619–628.

55. Rothblatt, J. A., and D. I. Meyer. 1986. Secretion in yeast: reconstitution of the translocation and glycosylation of α-factor and invertease in a homol-
genous cell-free system. *Cell.* 44:619–628.

56. Rothblatt, J. A., and R. Schekman. 1989. A hitchhiker's guide to analysis of the yeast invertase signal peptide: effects on protein transloca-

57. Rothblatt, J. A., and D. I. Meyer. 1986. Secretion in yeast: reconstitution of the translocation and glycosylation of α-factor and invertease in a homol-
genous cell-free system. *Cell.* 44:619–628.

58. Rothblatt, J. A., and R. Schekman. 1989. A hitchhiker's guide to analysis of the yeast invertase signal peptide: effects on protein transloca-

59. Rothblatt, J. A., and D. I. Meyer. 1986. Secretion in yeast: reconstitution of the translocation and glycosylation of α-factor and invertease in a homol-
genous cell-free system. *Cell.* 44:619–628.