In Vivo Evidence for Posttranslational Translocation and Signal Cleavage of the Killer Preprotoxin of *Saccharomyces cerevisiae*

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A full-length cDNA of the M1 double-stranded RNA killer preprotoxin coding region successfully directed the synthesis of secreted K1 toxin when expressed in *Saccharomyces cerevisiae* from a plasmid vector. Three protein species immunoreactive with antitoxin antiserum were detected intracellularly in transformants harboring this killer cDNA plasmid. These toxin precursor species were characterized by using secretory-defective hosts, by comparative electrophoretic mobilities, and by tunicamycin susceptibility. Such studies indicate that these three protein species represent intermediates generated by signal cleavage of the preprotoxin and its subsequent glycosylation and provide evidence that these events occur posttranslationally.

Posttranslational translocation of nascent polypeptides destined for export has been observed in procaryotes both in vivo (14, 24) and in vitro (20). In eucaryotes the requirement for coupling of translation and translocation appears to be more stringently observed, based on studies with cell-free systems (10, 31, 32). The signal recognition particle (SRP) is thought to facilitate the coupling of these two processes through the arrest of translation prior to docking on the microsomal membrane (32). Translational arrest by SRP has recently been shown to be less universal than originally thought, occurring only in the wheat germ system (17), which casts doubt on the stringency with which the coupling of translation and translocation actually occur.

In eucaryotes, the correct targeting of most exported proteins to the microsomal membrane relies on the interaction of SRP with the N-terminal pre- or signal sequence. In most instances the signal is thought to be proteolytically removed after cotranslational translocation of the protein across the membrane. The cotranslational model for translocation and signal cleavage predicts that detection of the unprocessed preprotein would be unlikely. Signal cleavage occurring posttranslationally has, however, been observed in vivo by detection of the unprocessed precursor for both the parathyroid hormone of rat (23) and a signal-defective acid phosphatase protein in *Saccharomyces cerevisiae* (11, 12). In both cases it was not clear whether the unprocessed forms were biologically significant. In this report we present evidence for the accumulation of a primary unprocessed precursor, of yeast killer toxin, and of its processed intermediates comprising the signal-cleaved and glycosylated forms.

Killer toxin is a secreted heterodimeric protein produced by strains of *S. cerevisiae* which contain a cytoplasmically inherited M1 double-stranded RNA (dsRNA) genome (for a review, see reference 27). This K1 toxin is synthesized as a precursor molecule with an N-terminal leader peptide and an interstitial glycosylated peptide separating the alpha and beta toxin subunits (3). Previously we have cloned and expressed a DNA copy of the killer toxin-coding region of the M1 dsRNA genome (16, 26). Analysis of the preprotoxin sequence revealed an N-terminal region approximately 26

amino acids long with typical signal sequence characteristics (22, 29, 30, 33, 34), including a putative signal cleavage site (3) nested within the 44-amino-acid leader peptide. When the region coding for the 44-amino-acid leader plus 8 amino acids of the alpha subunit was fused to bacterial cellulase, the leader efficiently directed secretion of the cellulase protein to the extracellular medium (25), suggesting that it has a signallike function.

Although the preprotoxin leader demonstrates targeting capability (25), evidence for signal cleavage is incomplete and conflicting. Evidence from in vitro protein synthesis studies with denatured M1 dsRNA or mRNA from wild-type killer strains argues that the toxin precursor undergoes signal cleavage (4). Evidence from in vivo studies with wild-type killer strains is limited and equivocal but has been interpreted to suggest that no cleavage of the signal occurs (6).

In this report we provide evidence that yeast cells transformed with a full-length cDNA coding for the preprotoxin produce three protein species precipitable with antitoxin antiserum that are processing intermediates and occur early in the secretory pathway. These findings support a post-translational mechanism for completion of translocation through the endoplasmic reticulum membrane and signal cleavage of the killer toxin precursor.

MATERIALS AND METHODS

Yeast strains and medium. The diploid *S. cerevisiae* T158c/S14a (ATCC 46427) was used as a typical K1 killer containing the M1 dsRNA genome (5). *S. cerevisiae* AH22 (a *leu2-3 leu2-112 his4-519*) was used as the recipient strain for transformation with the cDNA plasmid constructs pL308, pL70, and pL317. The temperature-sensitive secretion-defective mutants MHSF331 (a *sec53-6*) and HMSF176 (a *sec18*) were obtained from the Yeast Genetic Stock Center, University of California, Berkeley, and from R. Schekman, respectively. These temperature-sensitive nonkillers were crossed with C627-4B (α *ade1 leu2-2 leu2-112 his3-11 his3-15* [KIL-0]). The diploids were sporulated and asci were dissected. Spores with *leu2* and *ade1* markers were used for transformation with pL308.

Growth medium was the minimal medium described previously (16) supplemented as appropriate with 0.5% yeast extract, 0.5% peptone, and 2% glucose.

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FIG. 1. Immunoreactive precursor species in T158c/S14a and pL308 transformants of strain AH22. Cells were pulse-labeled for 10 min with [35 S]methionine, lysed, solubilized, and reacted with antitoxin antiserum (Ab) or preimmune serum (Pi) as described in the text. Samples were electrophoresed on SDS-polyacrylamide gels and visualized by fluorography. Sizes are indicated in kilodaltons.

cDNA constructs. A full-length DNA copy of the preprotoxin-coding region of M1 dsRNA (pL308) was constructed by using pADH10A (16). The 5' *Bam*HI-*Pst*I fragment was replaced with a synthetic oligonucleotide coding for the correct 5' sequence as described previously (2). The leader-deleted construct (pL317) was synthesized by removal of the 5' *Bam*HI-*Bgl*II fragment and ligation in frame with the start ATG with a *Bam*HI linker. The pL70 deletion construct, which eliminates the N-terminal 26 amino acids of the killer precursor leader and replaces them with a methionine and a leucine residue, was synthesized by replacing an *Hind*III-*Pst*I fragment with a smaller fragment made up of complementary oligonucleotides having the sequence 5'-AGCTTATG-CTG-AAC-GAT-GTG-GCC-GGT-CCT-GCA-3' and 3'-ATAC-GAC-TTG-CTA-CAC-CGG-CCA-GG-5'.

Yeast transformation with cDNA plasmids pL308, pL317, and pL70. Transformation into *S. cerevisiae* was done by the lithium procedure (13). Transformants were selected on minimal medium, pH 6, supplemented with the appropriate amino acids.

Immunoprecipitation of toxin and toxin precursors. Cultures were labeled with [35 S]methionine (Amersham Corp.) for approximately 10 min. Cells and medium were obtained by centrifugation. Growth medium was concentrated with ultrafiltration cones (CF-25; Amicon Corp.). Cells were lysed with glass beads, and the homogenates were solubilized with sodium dodecyl sulfate (SDS). Both cell lysates and concentrated medium were immunoreacted with antitoxin antiserum and protein A-Sepharose as described previously (5). Cells treated with the drug tunicamycin were incubated for 30 min at 30°C prior to pulse-labeling. Immunoprecipitated precursors were treated with endo- β -N-acetylglucosaminidase H (endo-H) overnight at 37°C as outlined by Trimble and Maley (28). The cell fractionation procedure consisted of cell breakage with glass beads followed by rapid centrifugation in a minifuge prior to solubilization with SDS. Pellet fractions were boiled in 0.1% SDS, diluted with reaction buffer, and immunoreacted as described above. Supernatant fractions were diluted with reaction buffer directly.

Immunoreactants were subjected to SDS-polyacrylamide gel electrophoresis and fluorography as described previously (5). Band intensities were estimated with a laser scanning densitometer coupled to an integrator.

In vitro translation of heat-denatured M1 dsRNA with rabbit reticulocyte lysate (Amersham Corp.) was done as described previously (5). After translation, the lysates were diluted in reaction buffer and immunoreacted with antitoxin antiserum.

Northern blot analysis. Total yeast RNA was extracted from strain T158c/S14a and AH22(pL308) transformants. Fixed amounts of RNA were applied to a formaldehyde gel, electrophoresed, and blotted, and baked filters were probed with [α - 32 P]dATP nick-translated probe containing the cDNA killer insert pKc-10A (26) and with pRB58 (7) as a control. Relative levels of killer mRNA were estimated by densitometer scanning of band intensities.

RESULTS

Precursor processing and toxin secretion in secretion-defective mutants. Immunoprecipitation of intracellular proteins after pulse-labeling of a host transformed with the cDNA killer expression vector pL308 indicated the presence of three immunoreactive species (Fig. 1). The largest of these three comigrated with the 42-kilodalton (kDa) intracellular glycosylated species normally observed in a pulse-labeled wild-type dsRNA-based killer strain, T158C/S14a. The estimated molecular size of the two smaller species, as determined from SDS-polyacrylamide gels, was 35 and 33 kDa. To attempt to identify the 35- and 33-kDa species, we examined their behavior in secretion-defective mutants. As described previously (6), processing of the wild-type killer preprotoxin is dependent on the normal yeast secretory pathway as defined by the temperature-sensitive secretion-defective mutants of Novick and Schekman (21).

With secretion-defective yeast mutants, it has been possible to demonstrate that the precursors in pL308 transformants accumulate at the endoplasmic reticulum and to establish precursor-product relationships between them. Strains for transformation were constructed by standard genetic techniques. Transformants with the relevant *sec* mutation and harboring the pL308 plasmid were grown at the permissive and restrictive temperatures, and the toxin precursors were analyzed after pulse-labeling and immunoprecipitation with antitoxin antiserum.

(i) **Endoplasmic reticulum translocation-defective host.** Under permissive conditions, the *sec53* host, defective in preprotein translocation into the endoplasmic reticulum, produced all three precursor species (Fig. 2A). Secretion of toxin occurred only at the permissive temperature (Fig. 2C). At the restrictive temperature, when translocation was arrested, only the two lower-molecular-weight species (33 and 35 kDa) were observed after pulse-labeling; after a chase of 30 min, only the 33-kDa species persisted (Fig. 2A, lanes 5 and 4). If chased longer than 30 min at the permissive temperature, all three species diminished in signal, as seen for the *sec18* precursors (Fig. 2B, lanes 3 and 2). These results localized the 35- and 33-kDa species to an early event in secretion, translocation across the endoplasmic reticulum membrane, and demonstrate a precursor-product relationship between them consistent with signal cleavage.

(ii) **Endoplasmic reticulum-accumulating host.** In a *sec18* mutant, which accumulates endoplasmic reticulum at the restrictive temperature, a different pattern of precursor accumulation was seen. As in the *sec53* mutant, all three precursors were detected after pulse-labeling at the permissive temperature (Fig. 2B, lane 3). At the restrictive temperature, in contrast to the *sec53* mutant, all three species remained detectable, but after a chase of 40 min the 35- and 33-kDa species diminished in intensity while the 42-kDa glycosylated species accumulated (Fig. 2B, lane 5). Again, secretion of toxin occurred only at the permissive temperature (data not shown). These pulse-chase data localized the 42-kDa species to the endoplasmic reticulum and correlated

the turnover of the 35- and 33-kDa precursors with the accumulation of the 42-kDa glycosylated species.

Characterization of the precursor species. Treatment of pulse-labeled T158c/S14a cells with tunicamycin, a drug which inhibits asparagine-linked core glycosylation, resulted in the accumulation of two novel immunoreactive species which comigrated with the two lower-molecular-weight species detected in pulse-labeled pL308 transformants (Fig. 3A, lanes 5 and 8). Following a 30-min chase of the tunicamycin-treated T158c/S14a cells, only the smaller species persisted (Fig. 3A, lane 6). Endo-H treatment of the immunoprecipitated T158c/S14a 42-kDa precursor resulted in the accumulation of a single species which migrated approximately with the 33-kDa pL308 precursor (data not shown). In tunicamycin-treated pL308 transformants, the 42-kDa species could not be detected, although the two lower-molecular-weight species were present (Fig. 3B, lane 3). Following a 60-min chase, the 33-kDa species was predominant (data not shown). No novel immunoreactive species were detected in these pL308 transformants following tunicamycin treatment. Endo-H treatment of the pL308 precursors resulted in the loss of the 42-kDa species and an increase in the detectable amount of the 33-kDa species (data not shown). These data suggest that the three immunoreactive species which accumulated in pL308 transformants were related to those detected in wild-type killer strains. The 42-kDa species was detectable under normal conditions, while the 35- and 33-kDa species could be detected only if T158c/S14a cells were treated with tunicamycin prior to pulse-labeling.

Comparison of electrophoretic mobilities with the primary translation product and two N-terminally truncated toxin precursors further suggested probable identities for the 33- and 35-kDa species. In vitro translation of the M1 dsRNA genome yielded full-length preprotoxin of estimated molecular size of 34,787 daltons (3, 4). Comparison of the electrophoretic mobilities of the primary translation product with the pL308 and T158c/S14a precursors after tunicamycin treatment revealed that the 35-kDa species comigrated in both cases (Fig. 3A, lanes 5, 7, and 8). This provides strong circumstantial evidence that this 35-kDa species is the in vivo preprotoxin. In one leader-deleted plasmid construct, pL317, in which all of the leader peptide and the three N-terminal amino acids of the alpha subunit were deleted, a single relatively stable unglycosylated precursor of approximate molecular size 30 kDa was detected in transformants after pulse-labeling and immunoprecipitation with antitoxin antiserum (Fig. 3C, lane 1). The calculated molecular size of the full-length pL317 precursor molecule, based on its nucleotide sequence, is 29,760 Da. The 33-kDa species seen in vivo in pL308 transformants migrated more slowly on SDS-polyacrylamide gels than this pL317 precursor (Fig. 3C). The second construct (pL70), which deleted 26 amino acids from the N-terminus of the leader when expressed in a transformed recipient strain, synthesized a single immunoreactive unglycosylated precursor after pulse-labeling (Fig. 3D, lane 3). This species comigrated with the 33-kDa species seen in vivo in pL308 transformants when run on an SDS-polyacrylamide gel (Fig. 3D, lanes 2 and 3). These data are consistent with the 33-kDa species being generated by signal cleavage within the 44-amino-acid leader, probably close to or at the predicted signal cleavage site between amino acids 26 and 27.

The half-lives of the three pL308 precursors were determined from pulse-chase data by densitometry scanning of fluorographs. The calculated values were averaged from

FIG. 2. Temperature-sensitive secretion-defective mutants (*sec53* and *sec18*) were transformed with pL308, and killer precursors were analysed after pulse-labeling at the permissive and restrictive temperatures. Cell lysates and concentrated medium were immunoprecipitated with antitoxin antiserum or with preimmune serum (Pi), electrophoresed, and visualized as described previously. Arrows indicate mobilities of precursor species. (A) Immunoreactive intracellular proteins in *sec53* host transformed with pL308, pulse-labeled for 10 min (Pi and 0' lanes) or followed by a 30-min chase (30' lanes) at the permissive (22°C) and restrictive (37°C) temperature. (B) Immunoreactive intracellular proteins in *sec18* host transformed with pL308, pulse-labeled for 10 min (Pi and 0' lanes) or following a 40-min chase (40' lanes) at the permissive (22°C) and restrictive (37°C) temperature. (C) Secreted proteins were concentrated from the medium following a 30-min chase at 22 or 37°C and immunoprecipitated with antitoxin antiserum.

three separate experiments at three time points. The 42-kDa glycosylated species had a half-life of approximately 15 to 20 min at 30°C. The 35- and 33-kDa species had half-lives of approximately 40 min.

Crude fractionation of intracellular precursors. Crude fractionation of pulse-labeled cells indicated that the largest proportion of all three of the observed precursors in pL308 transformants partitioned to the pellet (Fig. 4A). Cells transformed with the leader deletion construct pL317 showed a preferential partitioning of the precursor to the supernatant (Fig. 4B), consistent with reduced association of the mutant precursor with the endoplasmic reticulum. Twice as much material was used for the fractionation as was immunoprecipitated for the unfractionated samples.

Northern blot analysis. As an indirect measure of the relative levels of intracellular preprotoxin synthesis, mRNA levels in wild-type T158c/S14a cells and pL308 transform-

FIG. 3. Synthesis of immunoreactive precursors in wild-type and cDNA killer transformants. The *in vitro* translation product of denatured M1 dsRNA was included on some gels (lanes M1). Sizes are indicated in kilodaltons. (A) T158c/S14a cells were incubated with tunicamycin for 30 min at 30°C (+ lanes) or without tunicamycin (– lanes) pulse-labeled for 10 min (0' lanes) or chased for 30 min (30' lanes), and cell lysates were solubilized and immunoprecipitated with antitoxin antiserum or preimmune serum (Pi). Samples were then electrophoresed on SDS-polyacrylamide gels and visualized by fluorography. (B) Tunicamycin treatment of pL308 transformants of strain AH22. Samples were prepared as described for T158c/S14a (A). (C and D) AH22 transformants harboring either pL317, pL70, or pL308 were pulse-labeled for 10 min and immunoreacted with either antitoxin antiserum or preimmune serum (Pi lanes). Samples were then electrophoresed on SDS-polyacrylamide gels and visualized by fluorography. Arrows indicate 33 kDa.

ants were estimated by Northern blot analysis. pL308 transformants had approximately four times more mRNA than the T158C/S14a killers (data not shown), consistent with overproduction of the preprotoxin in pL308 transformants.

DISCUSSION

Expression in *S. cerevisiae* of a DNA copy of the yeast killer preprotoxin gene provides evidence for the intracellular accumulation of three proteins that are early processing intermediates. Multiple immunoreactive toxin precursors (Mp-1, Mp-1a, and Mp-1b) have previously been observed *in vitro* in an M1 dsRNA-primed reticulocyte lysate translation system in the presence of dog pancreatic microsomal membranes (4). Bostian et al. (4) proposed that the three species were generated by early events in secretion, that is, signal cleavage and core glycosylation. Our work supports this idea and further suggests that *in vivo* the multiple precursors which accumulate in pL308 transformants do so posttranslationally and represent the full-length primary translation product, preprotoxin (35 kDa), the signal-cleaved protoxin (33 kDa), and the N-linked core glycosylated protoxin molecule (42 kDa), which is normally detected in wild-type killers.

With secretion-defective yeast mutants, it has been possible to order some events in precursor processing for pL308 transformants at or before the endoplasmic reticulum. The preprotoxin accumulates and can be converted to a signal-cleaved species prior to complete entry into the endoplasmic reticulum at the *sec53* block and further to a glycosylated species in the *sec18*, endoplasmic reticulum-accumulating mutant. When translocation into the endoplasmic reticulum was blocked with the *sec53* mutant, pulse-labeling under restrictive conditions revealed accumulation of only two (35 and 33 kDa) of the three precursors. When a chase of 30 min followed pulse-labeling, only the 33-kDa species persisted. Two important conclusions follow from these observations.

First, the 35- and 33-kDa species accumulate prior to or during translocation into the endoplasmic reticulum. Second, there exists a precursor-product relationship (35 kDa → 33 kDa) between these two species consistent with a signal cleavage event.

When the endoplasmic reticulum-accumulating mutant (*sec18*) was pulse-labeled at the restrictive temperature, all three species were detected; but when chased for 40 min, the 35- and 33-kDa species diminished in intensity while the 42-kDa glycosylated species accumulated. These data define a relationship between two lower-molecular-size species and the 42-kDa precursor and establish the endoplasmic reticulum as the organelle in which the 42-kDa species is localized. The 42-kDa precursor normally detected in wild-type killer strains has previously been localized to the endoplasmic reticulum by using the *sec18* mutant (6).

Comparison of the precursors in pL308 transformants with (i) the T158c/S14a wild-type killers with and without tunicamycin and endo-H treatment, (ii) the *in vitro* primary translation product, and (iii) transformants harboring the leader-deleted plasmid constructs pL70 and pL317 established relative electrophoretic mobilities of the precursors consistent with the above scheme. The 42-kDa killer toxin precursor synthesized in the T158c/S14a killer strain comigrated with the 42-kDa species observed in pL308 transformants and shared tunicamycin and endo-H susceptibility. The primary translation product synthesized *in vitro* from an M1 dsRNA template comigrated with the 35-kDa species detected both in pL308 transformants and in tunicamycin-treated T158c/S14a. This result argues against posttranslational modification of the 35-kDa species and suggests that it is the preprotoxin. Comparison of the pL308 intracellular species with the leader-deleted precursors of the pL317 and pL70 transformants clearly demonstrated that the 33-kDa species was larger than the pL317 precursor by approximately 2 kDa and comigrated with the pL70 precursor. Removal of the entire leader peptide from the preprotoxin

would result in a loss of 44 amino acids, or approximately 5 kDa. It is unlikely that the pL317 precursor itself has been modified by cleavage, since its calculated molecular size, based on its nucleotide sequence, is 29,760 Da, which is in good agreement with that estimated from SDS-polyacrylamide gels (approximately 30 kDa). The conclusion to be drawn is that the 33-kDa species is probably derived from a cleavage event internal to the 44-amino-acid leader peptide, close to or possibly at the amino acid 26–27 junction predicted to be signal peptidase cleavage site (3). The calculated molecular size of the predicted signal-cleaved product is 31,859 Da. These results, while convincing, remain circumstantial and await definitive amino-terminal sequencing of the protoxin.

Bussey et al. (6) have shown that in vitro, localization of the toxin precursor to the lumen of the microsomal membrane vesicles (as assessed by glycosylation of the precursor and its stability to trypsin) was dependent on the cotranslational presence of these membranes in the system. This requirement does not necessarily reflect a coupling of translation and translocation of the preprotein across the membrane. Assuming that the SRP targeting model is applicable to *S. cerevisiae*, the SRP and SRP receptor interactions with the preprotein probably define the requirement for cotranslational addition of the membranes, as previously described for other secretory precursor proteins (18, 31). Mueckler and Lodish (19) have shown, however, that SRP can also interact posttranslationally with the human glucose transporter protein and that it facilitated insertion into the membrane. Lauffer et al. (15) postulate that the SRP precursor-SRP receptor complex additionally may facilitate the initiation of protein translocation through the endoplasmic reticulum. The half-life and fractionation data for the 35-kDa killer precursor argue that stable membrane association has occurred and that it may represent a putative "translocation-primed" molecule. Our in vivo and the earlier in vitro evidence for killer precursor processing in yeast (3) implies that targeting, docking, and translocation initiation events occur cotranslationally, but also suggests that signal cleavage and completion of translocation of the precursor can occur posttranslationally, as shown in Fig. 5.

The *sec53* mutation defines a posttranslational stage in the penetration of glycoprotein precursors into the endoplasmic reticulum (8, 9). Ferro-Novick and co-workers (8, 9) have estimated that translocation of the protein is arrested, so that the N-terminus penetrates the lumen of the endoplasmic reticulum. If any of the multiple precursor forms in the pL308 transformants represent processing intermediates either predocking or posttranslocation, a shift in the molecular sizes of some or all would be expected at the restrictive temperature. The fact that no changes were observed for either the 35- or 33-kDa species under these conditions suggests that both species accumulate after SRP docking but before core glycosylation, in other words, during translocation. Our evidence suggests that the 33-kDa species represents the signal-cleaved protoxin, and its accumulation at the restrictive temperature with a parallel decrease in the 35-kDa precursor indicates that signal cleavage occurs in a *sec53* blocked mutant. This result is consistent with preliminary findings described by Bernstein et al. (1), suggesting that secretory molecules accumulate on the luminal surface of the endoplasmic reticulum membrane when the *sec53* block is imposed.

Secretory precursor forms of invertase in *S. cerevisiae* cells treated with tunicamycin stick tightly to or become embedded in the endoplasmic reticulum (9). Here we show

FIG. 4. Crude fractionation of pulse-labeled proteins from pL308 (A) and pL317 (B) transformants into pellet (pel) and supernatant (sup). Cells were lysed as described in the text and subjected to rapid centrifugation in an Eppendorf microfuge for 5 min. The supernatant was removed, and the pellet was solubilized with SDS. Proteins associated with the respective fractions were analyzed by immunoprecipitation with antitoxin antiserum, electrophoresed on SDS-polyacrylamide gels, and visualized by fluorography. Samples in the last two lanes in both panels A and B were treated as described in the text and reacted with either antitoxin antiserum (Ab) or preimmune serum (Pi). The immunoprecipitated in vitro translation product of denatured M1 dsRNA (M1) is also shown in panel B.

that tunicamycin treatment of a T158c/S14a wild-type killer results in the accumulation of two precursor forms of the killer toxin which comigrate with the 35- and 33-kDa species detected in pL308 transformants and which were not resolved in previous work (6). Although fractionation experiments have not been done, these species are probably the same as the 35- and 33-kDa species in pL308 transformants. This accumulation may mark a bottleneck in the translocation apparatus of the endoplasmic reticulum, symptomatic of secretory apparatus perturbation either by drug treatment or by overproduction of the preprotein. Based on Northern blot analysis, elevated levels of preprotoxin synthesis are thought to occur in pL308 transformants. Haguenaer-Tsapir and Hinnen (11, 12) also observed accumulation of the acid phosphatase preprotein when yeast cells were transformed with the gene for this protein on a multicopy plasmid. They proposed that acid phosphatase precursor accumulation resulted from overproduction of the protein. Our results are consistent with this view and suggest that accumulation of intermediates is probably a consequence of overproduction of the preprotoxin and the saturation of the membrane translocation machinery of the endoplasmic reticulum.

With the criteria defined by Perlman and Halvorson (22) and von Heijne (29), a signal cleavage site was predicted to occur between Ala-26 and Leu-27 in the leader of the preprotoxin. The relative differences in size of the various precursors detected in pL308 transformants were consistent with this being the cleavage site (3). Presently we are

FIG. 5. Schematic representation of the proposed early processing events generating the three immunoreactive precursor species which accumulated in pL308 transformants. Symbols: ξ , signal sequence; $\bullet$, Asn residues; scissors, signal peptidase; Y, N-glycosidic oligosaccharide. E.R., Endoplasmic reticulum.

modifying this region by site-directed mutagenesis. With these mutants we hope to unequivocally determine the site of signal cleavage and define any possible function for the remaining leader fragment.

ACKNOWLEDGMENTS

We thank David Y. Thomas for his continuing interest in this project and for providing the pL308 and pL317 plasmid constructs. We also thank Dolores Harrison and Roland Brousseau for synthesizing the oligonucleotides.

This work was supported by operating and strategic grants from the Natural Sciences and Engineering Research Council of Canada.

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