Endocytobiosis & Cell Res. 6, 27-39 (1989)

BIOSYNTHESIS, PROCESSING AND MATURATION OF THIAMINE REPRESSIBLE ACID PHOSPHATASE OF Saccharomyces cerevisiae

Massoud Ramezani-Rad

Institut für Biochemie und Molekularbiologie, Freie Universität Berlin, D-1000 Berlin 33, FRG.

Key words: Acid phosphatase, processing, translocation, glycosylation, secretion, homologous system, yeast.

Summary: The thiamine repressible acid phosphatase (APase) Of Saccharomyces cerevisiae coded by the PHO3 gene is a cell surface glycoprotein. An in vitro translocation system has been reconstituted with subcellular fractions from the Saccharomyces cerevisiae. Pre-APase was faithfully translocated and glycosylated by yeast microsomes when present cotranslationally in the homologous cell-free system. Processed and not glycosylated APase was observed by addition of solubilized microsomal membrane to the translocation system.
Expression analysis of yeast transformed with PHO3-multicopy plasmid after treatment with tunicamycin shows accumulation of fully translated precursor of APase by pulse-labelling with (^{35}S)-methionine. Pulse-chase experiments resulted in the disappearence of unprocessed species and suggest the posttranslational translocation of pre-APase in these cells. Overproduction of secreted APase is seen in the cells harbouring the APase structural gene on a multicopy plasmid. Further results from the in vivo and in vitro experiments resulted in the identification of different intermediate forms of thiamine repressible acid phosphatase.

INTRODUCTION

The experiments of intracellular protein transport are taking a more important role in the area of the yeast molecular cell biology. The combination of biochemical analysis with genetic aproaches provides a unique opportunity to study the mechanism of the secretory pathway and membrane biogenesis in the yeast. The translocation of the newly synthesized secretory and membrane proteins through the endoplasmic reticulum is the result of the interaction between signal structures and membrane receptors. This mechanism has at first been studied

with the technique of fractionation and reconstitution of translocation activity ; secondly with the use of genetic analysis, e.g. the production of signal sequence mutants and the identification of essential genes for protein transport (reviewed in 1-3). After processing and core glycosylation the newly synthesized secretory proteins are transported to the Golgi apparatus and sorted to the target compartment. Complementary homologous reconstituted systems and the genetic approach lead to a more detailed description of the events in the secretory pathway.

In this report the novel correlation of in vivo data with in vitro results is shown, which enabled me to study the biosynthesis and maturation of thiamine repressible acid phosphatase (APase) in Saccharomyces cerevisiae. The phosphate metabolism of Saccharomyces cerevisiae is dependent on various acid and alkaline phosphatases, the expression of which is regulated from a complex control system at different levels. Orthophosphate deficiency induced the expression of at least two acid phosphatase genes; PHO5, PHO11 (4). The expression of PHO3 , a further acid phosphatase gene, is repressed by Thiamine (5). Genesequence analysis of PHO3 and PHO5 shows large homology both at the nucleotide (82%) and the amino acid (87%) level of these two tandemly linked genes. According to DNA sequence data, the PHO3 gene product has an 17-amino acid "signal" sequence at the N-terminus that is very similar to the signal peptide of the PHO5 gene product (6). We have recently described an in vitro assay for studying protein translocation in the yeast Saccharomyces cerevisiae (7,8,9). In this system, the yeast vacuolar protein prepro-carboxypeptidase Y was correctly co- and post-translationally translocated and glycosylated through the yeast microsomal membrane.

MATERIAL AND METHODS

Materials,Yeast Strains and Growth Condition:
[^{35}S]L-methionine (>800 Ci/mmol) was obtained from New England Nuclear; Protein A-Sepharose , PMSF, Triton X-100,

staphylococcal nuclease, EGTA, protein-molecular weight marker, Tunicamycin from Sigma; Ampicillin, Aprotinin, dNTPs, NTPs, pyruvate kinase, ribonuclease inhibitor, proteinase K, RNase A, restriction enzymes, from Boehringer-Mannheim; Zymolase 60T (60000 U/g) from Seikagaku Kogyo (Tokyo); Sephadex G-25 (medium) from Pharmacia; T4 DNA ligase,DNA polymerase, restriction enzymes from BRL Bethesda; Molecular Weight Markers SDS-6H and SDS-7B from SIGMA;
Saccharomyces cerevisiae strain SEY2202 (MATo leu2-3,ura 3-52 his4-519) provided by T.Stevens (University of Oregon, Oregon) was used for PHO3 multicopy plasmid transformation and RNA isolation. Diploid Saccharomyces cerevisiae strain RXII provided by A. Kotyk (Czechoslowenska Academy , Prag) and Schizosaccharomyces pombe strain h-972 from Leupold collection, were used for the preparation of the cell-free translation system and microsome membrane fraction. Growth of yeast cells for preparation of cell free system and microsomes was in 1% yeast extract, 2% peptone, 2% dextrose (YPD medium) to $1x10^7$ cells/ml. EMM2 (10) was used as thiamine free minimal medium.

Preparation of subcellular fraction:
For subfractionation cells were homogenized with french press or enzymatic digestion of cell wall.For spheroplasting, cells were harvested by centrifugation (1000 x g, 5 min, 25°C, washed with TS-buffer (50 mM Tris, 1.2 M sorbitol, pH 7.5) and resuspended at 50 units A_{600nm}/ml in 1.2 M sorbitol, 100 mM Tris/HCl pH 7.5, 10 mM $CaCl_2$, 200 µg/ml zymolase 60T and incubated at 30°C for 45 min. Spheroplasts were pelleted by centrifugation, resuspended at 20 units A_{600nm}/ml in 1.2 M sorbitol-YPD medium and incubated with gentle shaking for 30 min at 30°C. Spheroplasts were pelleted by centrifugation, washed in TS-buffer and resuspended in homogenisation buffer (20 mM Hepes/KOH, pH 7.5, 50 mM K-Acetate, 2 mM Mg-Acetate, 100 mM Sorbitol, 1 mM DTT, 1 mM PMSF).
The cell homgenisation was achieved by freezing and disruption in a french press cell (Aminco) as described (11) or submitting spheroplasts to 20 strokes of a tight-fitting pestle in a glass Dounce homogenizer on ice.
Preparationsof yeast cell-free translation extract and microsomal membrane fraction were as described previously (11,12).
Total cellular RNA from yeast strains was prepared as described (7) from PHO3-multicopy plasmid transformed yeast strains grown and derepressed in thiamine-free medium.

Construction of pDB248X/PHO3 Plasmid:
The hybrid yeast-Escherichia coli vektor pDB248X/PHO3 containing the PHO3 structural gene as an Pst1-BamH1 3-kilobase fragment was constructed from a PHO3 gene (promotor and structural gene) , which was isolated from plasmid pAP20 (13) and subcloned into pT712 (14). The plasmid pDB248X (15) was cleaved with HindIII and BamHI and was then ligated with 3-kb HindIII-BamHI fragment of pT712/PHO3.

Transformation:
Escherichia coli was transformed by the $CaCl_2$ method (16). Saccharomyces cerevisiae was
transformed by the Li-Acetate method (17).

Northern blot analysis:
Total yeast RNA was extracted from untransformed and transformed cells. Fixed amounts of RNA were applied to a formaldehyde gel; after electrophoresis and blotting, the baked filter was probed with [o $-^{32}$P]-dATP nick translated probe containing Pst1-BamH1 PHO3 fragment. Relative level of APase mRNA were estimated by densitometer scanning of band intensities.

Labelling of cells and immunoprecipitation:
Yeast cell transformants were grown overnight at 28°C in EMM2 medium, harvested at an A_{600nm} 0.6 , and suspended at an A_{600nm} 2.5 in fresh EMM2 medium. When indicated, tunicamycin treatment was then performed by 30 min of incubation at 30°C in the presence of 10 µg of tunicamycin per ml. Pulse labelling and pulse-chase labelling was performed by the addition of 100 µCi of [^{35}S]-methionine per ml and by incubation at 30°C. Pulse times and chase times were as indicated in the text. For subsequent chase, unlabelled L-methionine was added at a final concentration of 20 mM. At various time points aliquots of 250 µl radiolabelled samples were chilled and harvested. Cells were resuspended in 100 ul TN-buffer (150 mM NaCl, 50 mM Tris/HCl, pH 7.4, 1 mM DTT, 1 mM PMSF, 100 Kallikrein-units/ml Trasylol) and immediately broken by vortexing them with an equal volume of glass beads (diameter 0.5 mm) 6x15 sec with chill change.
Aliquots of radiolabelled samples boiled in 1% SDS were diluted 5 fold with Triton X-100 buffer and centrifuged twice for two min in a microfuge. The supernatant was removed, and then 15 µl packed Protein A-Sepharose was added to eliminate possible cross reactions. After 90 min incubation at 4°C, the supernatant was removed and processed for immunoprecipitation (18). Immunoprecipitated proteins were analysed by SDS-PAGE (19) and fluorography (20).

Acid phosphatase assay:
The measurement of acid phosphatase was essentially as described (21).

In vitro translation and translocation in the Saccharomyces cerevisiae cell-free system:
Total yeast RNA (4-10 µg) was translated at 20°C in a nuclease-treated cell-free yeast S48 system in a total volume of 25 µl in the presence of 20 µCi of [^{35}S]-methionine and in the presence or absence of microsomal membrane (0.1-0.5 units A_{280nm}). After 90 min incubation, the samples were denatured with 1% SDS for 4 min at 95°C. Detergent-solubilized translation reaction mixture was diluted with Triton X-100

buffer and immunoprecipitated.

Protease protection experiments:
Protease protection of translation mixture in the presence of microsomal membrane was carried out for 30 min on ice with Proteinase K at 500 µg/ml in the presence and absence of Triton X-100. Triton X-100 was used at 1%. The reaction was stopped by the addition of 1 mM PMSF, and after 5 min of incubation on ice the samples were denatured and immunoprecipitated.

RESULTS and DISCUSSION

I) Expression analysis of thiamine repressible acid phosphatase

The PHO3 gene was subcloned in a multicopy plasmid pDB248X, that carries the LEU2 gene. 3Kb PstI-BamHI fragment, which contains the total PHO3 gene, was recloned and used for the transformation of Saccharomyces cerevisiae cells. The transformant strains harboring PHO3-multicopy plasmid were assayed for transcription by Northern blot-analysis ; protein moiety by immunoprecipitation of in vivo products; and APase activity by enzyme test.
Untransformed and transformed cells grown in thiamine free minimal medium were harvested at mid-log phase and total RNA was extracted from each culture. Equal amounts of RNA from each preparation, as determined by A_{260nm} measurements, were fractionated by agarose gel eletrophoresis and transferred to nitrocelluose. Fig. 1A shows the result of hybridizing the membrane with [^{32}P]-PHO3 3Kb PstI-BamHI fragment labelled by nick translation. A single PHO3 transcript hybridizes in both preparations. The increase of gene copy number lead to a 3-5 fold increase of PHO3 mRNA level as estimated from densitometry measurements.
The cells were assayed for the presence of APase using antibody specific to APase followed by SDS-PAGE and fluorography. Fig. 1C shows that labelling with [^{35}S]-methionine for 10 min at 30°C of transformed strains indicates

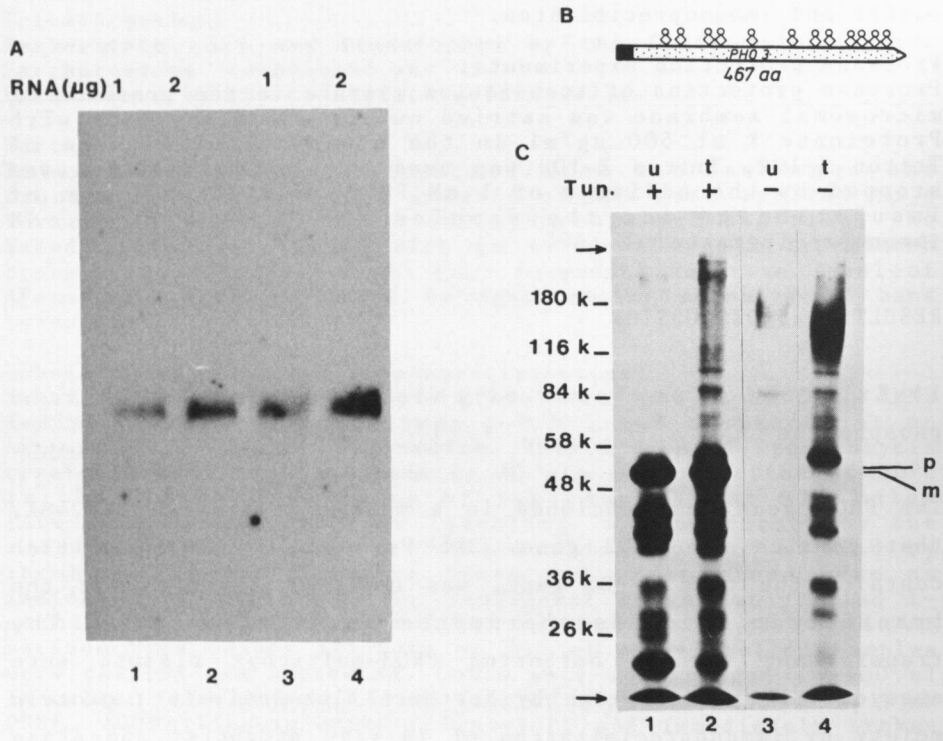

FIG. 1A. Northern blot analysis of total RNA prepared from untransformed (lane 1 and 2) and tranformed (lane 3 and 4) Saccharomyces cerevisiae cells grown in the absence of thiamine.
FIG. 1B. A linear map of pre-APase with positions of signal sequence and glycosylation sites.
FIG. 1C. Indirect immunoprecipitation of gene product of PHO3 with anti-APase. Untreated (-) and tunicamycin-treated (+) untransformed and transformed Saccharomyces cervisiae cells grown in minimal medium in the absence of thiamine, labelled with [^{35}S]-methionine and submitted to immunoprecipitation. Molecular weight markers are shown at the right of (C). p, precursor; m, mature protein.

Table 1. Synthesis of acid phosphatase in Saccharomyces cerevisiae cells carrying pDB248X/PHO3 and grown in the absence of thiamine.

strain	plasmid	cell no./ml $x10^7$	APase specific activity units/$2x10^7$ cells
SEY2202o	pDB248X/PHO3	2	3.88
SEY2202o	pDB248X/PHO3	5	3.71
SEY2202o	pDB248X	2	0.74
SEY2202o	pDB248X	6	1.01

an increase of immunoreactive APase species in comparison with untransformed strains. Transformed cells show also accumulation of unglycosylated precursor polypeptide and partially core glycosylated APase species (Fig. 1C lane 4). From these results it appeared that the production of PHO3 mRNA and protein in fully derepressed transformed strains probably leads to a partial saturation of some regulatory components at both transcription level and glycosylation level.

The enzyme activity was measured in intact yeast cells because the substrate p-nitophenyl phosphate passed through the cell wall. The APase activity increased about 4 times in the presence of pDB248X/PHO3 plasmid (Table 1). Transformed strain showed stronger APase activity at early log phase when grown on thiamine free minimal medium, whereas untransformed cells achieved a higher level of APase activity at late log phase. That means the derepression of the PHO3 gene in transformed cells is faster than the derepression of the untransformed cells under the experimental growth conditions.

II) Identification of precursor protein

The aminoterminal amino acid sequence of APase (PHO3 gene product), derived from the nucleotide sequence, shows the existence of signal peptide (6). The proteolytic processing

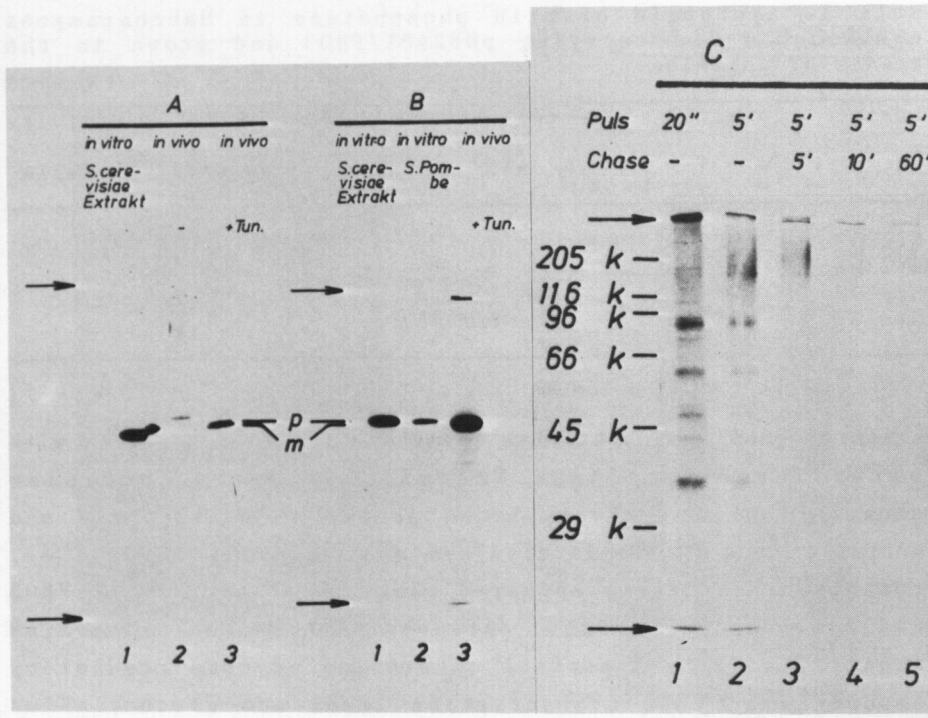

FIG. 2. Identification precursor protein of PHO3. Total RNA of transformed Saccharomyces cerevisiae cells grown in the absence of thiamin was translated in S48 cell-free system from Saccharomyces cerevisiae and Schizosaccharomyces pombe. In vitro translated proteins were submitted to immunoprecipitation, followed by SDS-PAGE and fluorography (FIG.2B lane 1 and 2). Saccharomyces cerevisiae cells grown in the absence of thiamine, were treated by tunicamycin (+) and labelled in vivo with [^{35}S]-methionine. Labelled cells were submitted to immunoprecipitation (FIG.2A and 2B lane 3). p, precursor; m, mature protein.

FIG. 2C. Immunoprecipitation of acid phophatase after pulse and pulse-chase labelling of Saccharomyces cerevisiae cells transformed with PHO3 multicopy plasmid. Saccharomyces cerevisiae cells grown in minimal medium under thiamin repression and submitted to labelling for the time indicated above each lane in the presence of [^{35}S]-methionine, followed by a Chase. Molecular weight markers are shown at the right of (C).

and glycosylation of thiamine repressible acid phosphatase was analysed through the comparison of in vitro and in vivo translation products (Fig. 2).
The immunoprecipitation of in vivo labelled proteins of pDB248X/PHO3 transformed cells in the presence of tunicamycin indicates the presence of two immunoreactive species (Fig. 2A;2B lane 3). The smaller species has the estimated molecular size of processed and unglycosylated species normally observed in a pulse labelled wild type strain. The larger comigrates with the 57 KD in vitro primary translation product, suggesting that transformed cells are able to translate PHO3 messenger in the absence of glycosylation.
The immunoprecipitation of pulse labelled proteins of pDB248X/PHO3 transformed cells and subsequent chase (Fig. 2C) shows the disappearence of unglycosylated, core glycosylated and incompletely core glycosylated species. These results suggest that transformed cells are able to translocate posttranslationally the in vivo primary translation product.

III) In vitro translocation and glycosylation of thiamin inducible APase

Microsomal membrane from Saccharomyces cerevisiae were prepared from postmitochondrial supernatant as explained previously (7) and treated with micrococcal nuclease to remove RNA. They were used for translocation of preAPase synthesized in a cell free translation system derived from Saccharomyces cerevisiae (11). Total RNA was isolated from PHO3-multicopy plasmid transformed Saccharomyces cerevisiae cells. Fig. 3 shows the translation products of these RNAs in homologous cell free system after immunoprecipitation with anti-APase, SDS-PAGE and fluorography. In the absence of microsomes the 57 KD primary translation product of APase was found (Fig. 3A lane 1 and Fig. 3B lane 1). Addition of solubilized yeast microsomes shows a hazy double band (Fig. 3A lane 2). These results, together with in vivo results (Fig. 2), indicate the

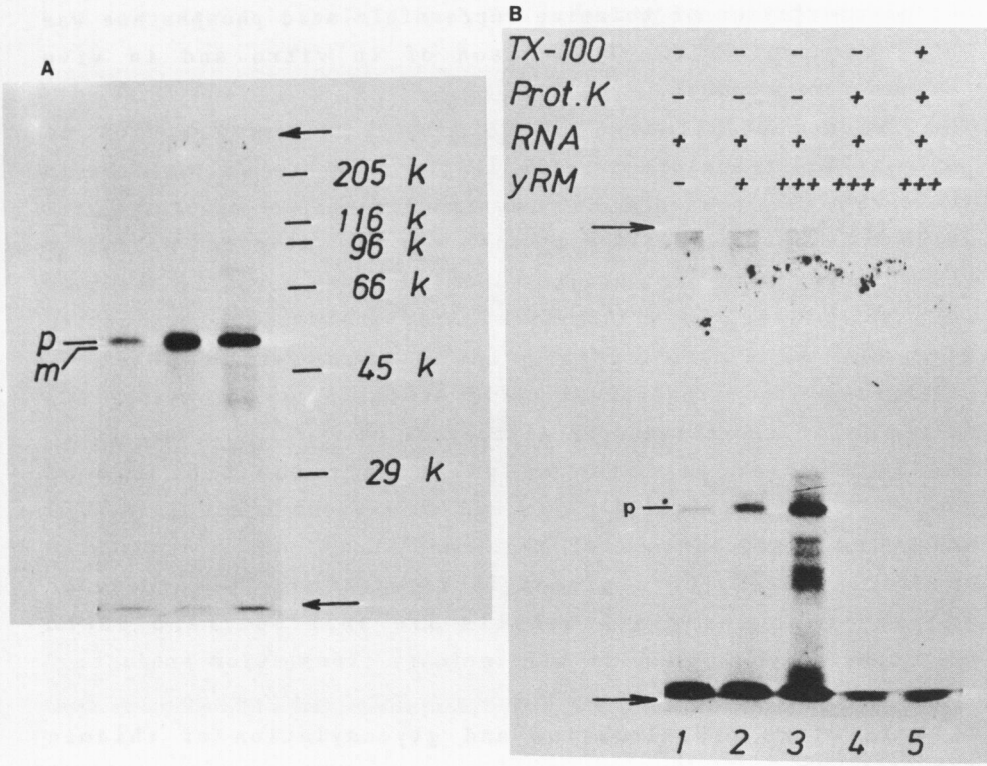

FIG. 3. In vitro translocation and core glycosylation of thiamine repressible APase. Translation was carried out in a final reaction volume of 25 ul with total RNA of transformed Saccharomyces cerevisiae cells grown in the absence of thiamin in a nuclease treated Saccharomyces cerevisiae S48 exract.
FIG.3A. shows immunoprecipitation of in vitro translated pre-APase (p) in Saccharomyces cerevisiae S48 extract (lane 1); in vitro translation product in the presence of solubilized yeast microsomes (treated with 1% <W/V> Triton X-100) (lane 2); in vitro translation product in the presence of intact nuclease treated yeast microsomes (lane 3). Molecular weight markers are shown at the left of (A).
FIG. 3B. Microsomes were added to a translation as in FIG. 3A lane 1 (lane 1) at 0.1 unit A_{280nm}/25 µl assay (lane 2) and 0.3 unit A_{280nm}/25 µl assay (lane 3). After incubation, aliquots were subjected to the proteinase K protection experiment with a final concentration of 100 µg/ml proteinase K (lane 4) and 1% Triton X-100 (lane 5).

signal peptide cleavage and processing of the primary translation product. Addition of intact microsomes show extra bands up to 80 KD, corresponding to partially core glycosylated and core glycosylated forms of precursor (Fig. 3A lane 3 and Fig. 3B lane 2-4). The relative abundance of extra bands was dependent on the microsome concentration. At low concentration (0.1 unit A_{280nm}/25µl assay) the bands with low Mr polypeptides were abundant (Fig. 3B lane 2). At high membrane concentration (0.3 unit A_{280nm}/25µl assay) the bands with higher Mr polypeptides were present (Fig. 3B lane 3). The higher Mr species of membrane specific polypeptides were protected from degradation by proteinase K (Fig. 3B lane 4). Addition of the non-ionic detergent Triton X-100 to destroy the integrity of the membrane barrier resulted in proteolysis of the formerly protected proteins (Fig. 3B lane 5). These in vitro data show that preAPase is translocated and glycosylated if microsomal membranes of Saccharomyces cerevisiae are present during translation. Glycosylation did not occur posttranslationally in the presence of puromycin and cycloheximide (results are not shown).
These novel properties of thiamin repressible acid phosphatase gene of Saccharomyces cerevisiae support its suitability as a new expression vector.

CONCLUSION

Correlation of in vivo data with in vitro results has given me the opportunity to study the biosynthesis and maturation of thiamin repressible acid phosphatase in Saccharomyces cerevisiae. The thiamin repressible acid phosphatase of Saccharomyces cerevisiae coded by the PHO3 gene is a cell surface glycoprotein. It was shown that pre-APase was cotranslationally translocated and glycosylated in homologous cell-free system. The addition of solubilized microsomal membrane to the cell-free system leads to processed but not glycosylated APase. Expression analysis of yeast transformed

with PHO3-multicopy plasmid after treatment with tunicamycin shows accumulation of fully translated precursor of APase by pulse-labelling with [^{35}S]-methionine. Pulse-chase experiments resulted in the disappearence of unprocessed species and suggests the posttranslational translocation of pre-APase in these cells.

Overproduction of secreted APase is seen in the cells harbouring the Apase structural gene on a multicopy plasmid.

Further results from the in vivo and in vitro studies has shown that the following intermediate forms are seen for thiamin repressible APase: The primary translation product (pre-APase) is about 57 KD in SDS-PAGE system. This product appeared as well in vitro translation system as in vivo only transformed cells. The processed and not glycosylated product is about 2 KD smaller in SDS-PAGE system. Many core glycosylated intermediate forms up to 80 KD are observed in vivo as well as in vitro. The mature APase is to identify between 120 to 220 KD at the cell surface.

Taken together with previous reports on 1) import of proteins into chloroplasts, mitochondria, and peroxysomes (glyoxysomes), 2) export of proteins from bacteria, and 3) translocation of finished polypeptide chains through the endoplasmic reticulum - at least in yeast (22) - these results imply that proteins, destined to be translocated across membranes, share in a basic cellular process present in all cells.

Acknowledgements: I am grateful to Dr. E. Schweingruber for kindly providing the antibody against APase, Dr. N. Kaeufer, Dr. R. Kramer and Dr. P. Nurse for plasmids pAP20 and pDB248X.

REFERENCES

1. Wickner, W. T. & Lodish, H. F. (1985): Science 230,400-407.
2. Walter, P., & Lingapaa, V. R. (1986): Ann. Rev. Cell Biol. 2, 499-516.
3. Scheckman, R. (1985): Ann. Rev. Cell Biol. 1, 115-143.
4. Sengstag, C. & Hinnen, A. (1987): Nucleic Acid Res. 15, 233-246.

5. Schweingruber, M. E., Flur, R., Maundrell, K., Schweingruber, A.- M., & Damermuth, E. (1986): J. Biol. Chem. 261, 15877-15882.
6. Bajwa, W., Meyhack, B., Rudolph, H., Schweingruber, A.-M., & Hinnen, A. (1984): Nucleic Acid Res. 12, 7721-7739.
7. Ramezani-Rad, M., Kaeufer, N., Hasilik, A. & Lochmann, E.R. (1985): Endocyt. Cell Res. 2, 249-260.
8. Ramezani-Rad, M. (1987): Ph. D. thesis, Free University of Berlin, West-Berlin.
9. Ramezani-Rad, M., Katz, H., Seyfarth, A. (1987): Biol. Chem. Hoppe-Seyler's 368,1267.
10. Mitchison, J. M. (1970): Methods Cell Phisiol. 4, 131-165.
11. Swida, U., Kreutzfeldt, C., Ramezani-Rad, M. & Kaeufer, N. (1982): FEMS Microbiol. Lett. 15, 313-318.
12. Kreutzfeldt, C. & Lochmann, E.-R. (1983): FEMS Microbiol. Lett. 16, 179-182.
13. Anderson, N., Thill, G. P. & Kramer, R. A. (1983): Mol. Cell Biol. 3, 562-569.
14. Bethesda Research Laboratories (1985): Focus 7, 9-10.
15. Beach, D. & Nurse, P. (1981): Nature(Lond.) 290, 140-143.
16. Broun, M. G. M., Weston, A., Saunders, J. R. & Humphreys, G. O. (1979): FEMS Microbiol. Lett. 5, 219-222.
17. Ito, H., Fukuda, Y. Murata, K. & Kimura, A. (1983): J. Bacteriol. 153, 163-168.
18. Anderson, D. I. & Blobel, G. (1983): Methods Enzymol. 96, 111-120.
19. Laemmli, U. K. (1970): Nature(Lond.) 227, 680-685.
20. Bonner, W. M. & Laskey, R. A. (1974): Eur. J. Biochem. 46, 83-88.
21. Toh-e, A., Inouye, S. & Oshima, Y. (1981): J. Bacteriol. 145, 221-232.
22. Verner, K., Schatz, G. (1988): Science 241, 1307-1313.

Received: October 3, 1988

Accepted: May 8, 1989