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Vesicular Transport of Extracellular Acid Phosphatases in Yeast *Saccharomyces cerevisiae*

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Abstract—A method for isolation of secretory vesicles from the yeast *Saccharomyces cerevisiae* based on the disintegration of protoplasts by osmotic shock followed by separation of the vesicles by centrifugation in a density gradient of Urografin was developed in this study. Two populations of the secretory vesicles that differ in density and shape were separated. Acid phosphatases (EC 3.1.3.2) were used as markers of the secretory vesicles. It was shown that the constitutive acid phosphatase (*PHO3* gene product) is mainly transported to the cell surface by a lower density population of vesicles, while the repressible acid phosphatase (a heteromer encoded by *PHO5*, *PHO10*, and *PHO11* genes) by a vesicle population of higher density. These data provide evidence that at least two pathways of transport of yeast secretory proteins from the place of their synthesis and maturation to the cell surface may exist. To reveal the probable reasons for transport of Pho3p and Pho5p/Pho10p/Pho11p enzymes by two different kinds of vesicles, we isolated vesicles from strains that synthesize the homomeric forms of the repressible acid phosphatase. It was demonstrated that glycoproteins encoded by the *PHO10* and/or *PHO11* genes could be responsible for the choice of one of the alternative transport pathways of the repressible acid phosphatase. A high correlation coefficient between bud formation and secretion of Pho5p phosphatase and the absence of correlation between bud formation and secretion of minor phosphatases Pho10p and Pho11p suggests different functional roles of the polypeptides that constitute the native repressible acid phosphatase.

Key words: yeast, *Saccharomyces cerevisiae*, secretion, transport vesicles, sorting, budding, acid phosphatases

Precise transport of the newly synthesized proteins to different organelles or secretion from the cell is an essential condition of the normal functioning of any eucaryotic cell.

A well-established scheme of the transport of secreted proteins in yeast was mainly worked out by Schekman et al. [1]. The secretory proteins in eucaryotes are transported by an exocytosis pathway in specialized vesicles. It was supposed until recently that the secreted proteins in yeast did not possess any sorting signal and were delivered to the cytoplasmic membrane towards the forming bud in a "bulk flow" [2]. In addition, mutant vacuolar proteins without a specific sorting signal lost the ability to be delivered precisely to the vacuole and were transported to the cytoplasmic membrane [3]. During recent years a number of studies were done where the authors managed to distinguish between two types of secretory vesicles in the "bulk flow"; these two types of vesicles differed in density and the set of secretory and membrane proteins [4, 5]. However, it was not established in these studies how did the distribution of different proteins between two types of vesicles occurs.

The well-known extracellular proteins, acid phosphatases, are useful markers for the study of the sorting and transport of secretory proteins in the yeast *Saccharomyces cerevisiae*. Two forms of acid phosphatases are known in this species, a so-called constitutive form encoded by the *PHO3* gene, and a repressible form encoded by three genes—*PHO5*, *PHO10*, and *PHO11*. Both phosphatases are glycoproteins with the carbohydrate part of the molecule constituting 50% of the molecular weight [6]. Phosphatases possess quaternary structure (form tetramers) [7]; the repressible acid phosphatase is a heteromer in which two minor polypeptides Pho10p and Pho11p represent 15% of the molecular weight [8]. These data suggest that not all molecules of the repressible acid phosphatase contain the minor polypeptides. The organization of the quaternary structure of these enzymes is not yet studied, and the role of individual components in the formation and behavior of the heteromer remains

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obscure. The expression of the *PHO3* gene occurs at high concentration of inorganic phosphate in the medium, while the expression of the repressible acid phosphatase occurs only during phosphorus starvation. At the same time in the absence of the inorganic phosphate in the medium, when the expression of the repressible acid phosphatase is stimulated, the synthesis of the constitutive acid phosphatase is repressed [9]. Consequently, the expression of the repressible or the constitutive acid phosphatases could be switched on by regulation of the concentration of the inorganic phosphate in the medium in strains that possess corresponding structural genes. It was demonstrated earlier [10] that a high correlation existed between bud formation and the secretion of the constitutive acid phosphatase, but there was no correlation between budding and the secretion of the repressible acid phosphatase. A suggestion based on these data was that yeast could possess an alternative secretory transport pathway for the extracellular proteins. Two transport pathways for excretory proteins are well known in higher eucaryotes, constitutive and regulated [11].

The goal of this study was to clarify the existence of two pathways for secretory proteins in yeast. For this a new method of the isolation of transport vesicles is proposed because existing methods [12-14] have severe drawbacks. In fact, mechanical disintegration of protoplasts leads to their partial destruction, and sucrose medium favors the destruction of the vesicles [15]. The native repressible and constitutive forms of acid phosphatase, as well as homomeric forms of acid phosphatase were used as the markers of the secretory vesicles. The strains synthesizing homomeric phosphatase forms were constructed earlier [8].

The correlation between the formation of buds and the secretion of the homomeric forms of acid phosphatase was determined simultaneously [16].

MATERIALS AND METHODS

Strains and plasmids. The strains used in this study are presented in Table 1. Plasmid pDP34-GAPFEL-*PHO5* was kindly provided by Dr. Hinnen (Hans-Knoll Institute of Natural Substances Research, Jena, Germany).

Conditions of cell cultivation and phosphatase synthesis. The cells were grown in medium containing 2% (w/v) bactopeptone, 1% (w/v) yeast extract (Difco, USA), 6% (w/v) glucose (Merck, Germany), 10 mM KH_2PO_4 in 0.02 M succinate buffer (Reanal, Hungary), pH 5.0 (medium rich in inorganic phosphate).

To obtain medium free of phosphate, inorganic phosphate was eliminated using $CaCl_2$ [17].

The abbreviations of acid phosphatases together with corresponding genes used in this article are given in Table 2.

To obtain the homomeric phosphatase A, yeast cells of YMR4 strain were transformed with the plasmid pDP34-GAPFEL-*PHO5*; the transformed cells provided the expression of the *PHO5* gene that was independent of the concentration of inorganic phosphate. The cells were grown in the medium with phosphate.

Table 1. *Saccharomyces cerevisiae* strains that were used in this study

Strain	Genotype	Source
S 288C	a MAL, GAL2	P. V. Venkov (Bulgaria)
YMR4	α his 3–11,3-15 leu 2–3, 2–112 can^R pho5-pho3::ura3Δ1	A. Hinnen (Germany)
YMR44	α his 11, 3–15 leu 2–3, 2–112 can^R pho5-pho3::ura3Δ1, ura3Δ5 pho11::URA3	M. G. Shnyreva (USA)
YMR46	α his 11, 3–15 leu 2–3, 2–112 can^R pho5-pho3::ura3Δ1 ura3 pho10::URA3	M. G. Shnyreva (USA)

For expression of the homomeric phosphatases B and C the cells of YMR44 and YMR46 strains were grown in the medium without phosphate.

To express the native repressible acid phosphatase, yeast cells of S288C strain were grown in the medium without phosphate, while the native constitutive acid phosphatase was expressed during growth of cells of the same strain in the medium with phosphate.

Isolation of secretory vesicles. The cells were grown until the early exponential phase ($A_{600}^{1\,cm} = 0.7\text{-}1.0$) at 30°C in medium with or without inorganic phosphate, sedimented by centrifugation at 2000*g* for 10 min and washed with buffer containing 0.8 M sorbitol (Serva, Germany) and 30 mM dithiothreitol (DTT) (Reanal) in 0.15 M solution of sodium citrate, pH 6.0 (buffer A). The cells were suspended in the same buffer and incubated 15 min at room temperature.

The cells were washed with buffer A without DTT and incubated with the enzyme cytohelicase (IBF Biotechnics, France) using 6 ml of buffer A for 1 g of wet cells at final enzyme concentration 8 mg/ml at 30°C for 30 min. The resulting protoplasts were pelleted at 1000*g* for 10 min and were washed twice with buffer A. The washed protoplasts were subjected to osmotic shock in hypotonic solution containing 8% Ficoll 400 (Pharmacia, Sweden) in 0.1 M sorbitol solution in 0.025 M Tris-HCl buffer, pH 7.2 (Trizma Base, Sigma, USA).

The resulting material was incubated 2 h on ice, then centrifuged at 10,000*g* for 20 min to eliminate heavy organelles, cytoplasmic membrane, and Golgi membranes. The supernatant containing microsomal fraction, vacuoles, and soluble proteins was subjected to centrifugation in a density gradient.

Table 2. Designation of acid phosphatases

Acid phosphatase	Expressed genes
A	PHO5
B	PHO10
C	PHO11
Repressible acid phosphatase	PHO5, PHO10, PHO11
Constitutive acid phosphatase	PHO3

The solution of 76% Urografin (Scherring, Germany) diluted to the specific density 1.06 and 1.18 g/cm^3 with 0.1 M sorbitol solution in 0.025 M Tris-HCl buffer, pH 7.2, was used for the linear density gradient. Equal volumes (3 ml) of these solutions were put in the gradient mixer (LKB, Sweden), and a linear gradient was formed in ultra transparent centrifuge tubes for Beckman SW-41 rotor. A 4-ml sample of the studied supernatant, then successively 1 ml of 8% Ficoll 400 in 0.1 M sorbitol solution in 0.025 M Tris-HCl buffer, pH 7.2, and 1 ml of 6% Ficoll 400 in 0.1 M sorbitol solution in 0.025 M Tris-HCl buffer, pH 7.2, were overlaid on the formed gradient. The tubes with samples were centrifuged in a Beckman L7-55 ultracentrifuge (USA) in a Beckman SW-41 rotor at 110,000*g* for 2 h. Two fractions of vesicles obtained after centrifugation (with lower specific density, L-vesicles, and with higher specific density, H-vesicles) were collected using a syringe with curved needle, diluted with 0.3 M sorbitol solution in 0.025 M Tris-HCl, pH 7.2, and were sedimented in a Beckman SW-41 rotor at 150,000*g* for 30 min. The resulting vesicles were used for subsequent studies.

Other methods. The enzymatic activity of acid phosphatases was measured using 4-nitrophenylphosphate (Sigma) as the substrate [18]. Before the measurement of the activity the solution of Triton X-100 (LKB) was added to the suspension of vesicles to the final concentration 1%.

To obtain the electron microphotographs, the vesicles were negatively stained with phosphotungstic acid [19]. The samples were examined using a JEM-100B electron microscope (JEOL, Japan) at 80 kV.

Protein was measured according to the method of Lowry [20], and orthophosphate by the method of Panusz [21].

RESULTS AND DISCUSSION

A high correlation between bud formation and the secretion of the constitutive acid phosphatase and the absence of such correlation in the case of the repressible acid phosphatase was demonstrated in the study of Shnyreva and Egorov [10]. They suggested that in yeast, like in higher eucaryotes, there are two secretion pathways—constitutive and regulated.

Two approaches were applied in this study to test this suggestion. First, the correlation was established between bud formation and secretion of the marker enzymes in the medium. Secondly, the distribution of the enzymatic activity between fractions of secretory vesicles was studied.

The secretion of homomeric forms of phosphatases was studied to elucidate the possible role of individual subunits of the native repressible acid phosphatase in the choice of the secretion pathway of the mature enzyme. The correlation between two series of data, bud quantity in 1 ml and corresponding growth of the total extracellular activity of each of three homomeric enzymes (total activity both associated with the cells and in the medium for 1 ml of suspension) was analyzed [16]. A high correlation coefficient indicates that the total growth of the enzymatic activity is in proportion with the bud quantity in the culture, i.e., the enzyme secretion occurs through the constitutive transport pathway through buds. It was suggested earlier [22] that all extracellular yeast enzymes are secreted only through this pathway. As can be seen from Table 3, the secretion of the homomeric acid phosphatase A has a high level of correlation with budding, i.e., the enzyme is secreted through the constitutive pathway. The secretion of the homomeric acid phosphatases B and C did not correlate with bud formation. Since the secretion of the repressible acid phosphatase also does not correlate with budding, it can be suggested that just the presence in this homomeric enzyme of the minor components B and C determines the alternative pathway of secretion.

To determine the causes of different delivery of the acid phosphatases to the cell surface, it seemed useful to analyze their content in different types of secretory vesicles that mediate their transport.

Using the approach of Shabalin and Kulaev [23], we elaborated a method of mild isolation of secretory vesicles based on protoplast destruction by osmotic shock followed by separation of vesicles in a density gradient of Urografin. All isolation steps were carried out at 4°C except in specially indicated cases.

This method was applied to obtain vesicles from strain S288C that was grown in the medium rich in inorganic phosphate and in the medium without phosphate. After separation in the density gradient of Urografin of the microsomal fraction that did not pellet at 10,000*g*, we obtained two fractions of vesicles (Fig. 1, a and b). During the cell growth in the medium without phosphate the fraction with higher density (H-vesicles, density 1.13 g/cm^3) contained 65% of the total protein of the vesicles, while the fraction with lower density (L-vesicles, density

Table 3. Relationship between budding and secretion of extracellular acid phosphatases

Strain	Acid phosphatase	Inorganic phosphate in the medium	Correlation coefficient	% of phosphatase activity in the secretory vesicles	
				light vesicles	heavy vesicles
S288C	Native constitutive acid phosphatase	+	0.72	66	34
S288C	Native repressible acid phosphatase	—	0.21	28	72
YMR4 pDP34-PHO5	Phosphatase A	+	0.90	77	23
YMR44	Phosphatase B	—	0.08	36	64
YMR46	Phosphatase C	—	0.16	28	72

1.09 g/cm^3) constituted 35%. During the growth in the medium rich in inorganic phosphate the inverse distribution was observed: about 70% of protein was in the L-vesicle fraction and about 30% in the H-vesicle fraction.

Electron microphotographs demonstrate (Fig. 2, a and b) that the two types of vesicles differ in electron density and in the shape of the surface and have average diameter 100-150 nm. The surface of H-vesicles is granular, while the surface of L-vesicles is smooth. The diameter of isolated vesicles is slightly higher than those observed in thin layers of intact yeast cells (80-100 nm [24]).

The vesicles isolated from the cells grown both in the medium with inorganic phosphate and without it were

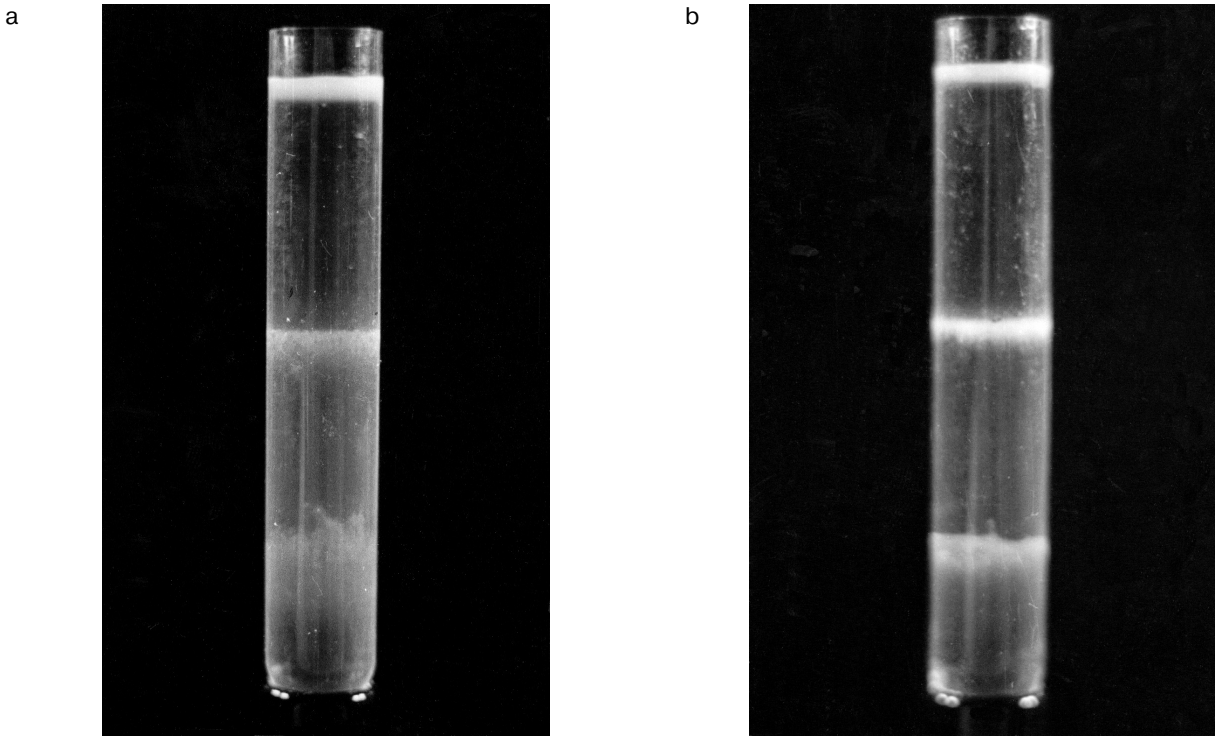

Fig. 1. Centrifuge tubes with secretory vesicles after separation in the density gradient of Urografin: a) vesicles from the cells of S288C strain after cultivation in the medium rich in inorganic phosphate; b) vesicles of the same strain after cultivation in the medium without phosphate. The upper layer, vacuoles; the middle layer, L-vesicles; lower layer, H-vesicles.

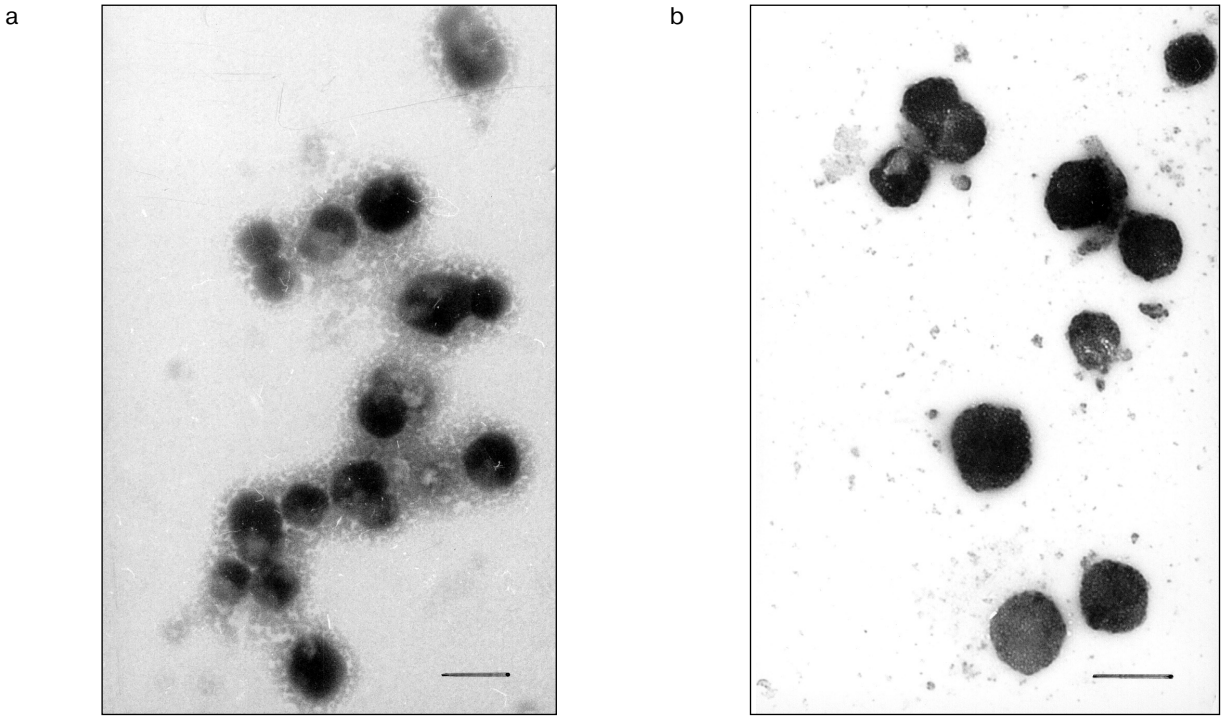

Fig. 2. Electron microphotographs: a) L-vesicles; b) H-vesicles. Bar, 200 nm.

used for the measurement of the phosphatase activity. In the first case the major part of the activity was concentrated in the L-vesicles (constitutive acid phosphatase), in the second case in the H-vesicles (repressible acid phosphatase) (Table 3).

These data suggest the existence of the system of protein sorting in the secretory pathway: some proteins are delivered to the cell surface through the constitutive pathway, while others are transported by a different pathway.

To elucidate what factors may determine the sorting, the vesicles from the strains that synthesize homomeric acid phosphatases A, B, and C were isolated. The results of this study demonstrated that the homomeric acid phosphatase A was transported to the cell surface mainly in the L-vesicles (Table 3). This means that the *PHO5* gene product is not able to select the alternative pathway of secretion and does not differ in this property from the *PHO3* gene product (constitutive acid phosphatase). The phosphatases B and C are mainly transported in the H-vesicles (Table 3). Since the repressible acid phosphatase consists of the expression products of three genes and is transported in the H-vesicles, it can be suggested that notably minor components of the repressible acid phosphatase (*PHO10* and/or *PHO11* gene products) are responsible for the transport of the native enzyme in the vesicles of high density through the alternative pathway.

Thus, the data obtained in this study provide evidence that yeast possesses two secretion pathways, like higher eucaryotes. The new method for isolation of transport vesicles may serve as a useful tool for further studies of the mechanisms of sorting and transport of secretory proteins.

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REFERENCES

1. Schekman, R., and Novick, P. (1982) in *Molecular Biology the Yeast Saccharomyces cerevisiae. Metabolism and Gene Expression* (Strathern, J. N., Jones, E. W., and Broach, J. R., eds.) Cold Spring Harbor Laboratory Press, New York, pp. 361-398.
2. Pryer, N. K., Wuestehube, L. J., and Schekman, R. (1992) *Ann. Rev. Biochem.*, **61**, 471-516.
3. Bryant, N. J., and Stevens, T. H. (1998) *Microbiol. Mol. Biol. Rev.*, **62**, 230-247.
4. Harsay, E., and Bretscher, A. (1995) *J. Cell Biol.*, **131**, 297-310.
5. Lupashin, V. V., Kononova, S. V., Ratner, Y. N., Tsiomenko, A. V., and Kulaev, I. S. (1992) *Yeast*, **8**, 157-169.
6. Kozulic, B., Barbaric, S., Ries, B., and Mildner, P. (1984) *Biochem. Biophys. Res. Commun.*, **122**, 1083-1090.
7. Shnyreva, M. G., Tsuprun, V. L., Stel'mashchuk, V. Ya., and Egorov, S. N. (1992) *Biokhimiya*, **57**, 762-768.

8. Shnyreva, M. G., Petrova, E. V., Egorov, S. N., and Hinnen, A. (1996) *Microbiol. Res.*, **151**, 291-300.
9. Tait-Kamradt, A. G., Turner, K. J., Kramer, R. A., Elliott, Q. D., Bostian, S. J., Thill, G. P., Rogers, D. T., and Bostian, K. A. (1986) *Mol. Cell Biol.*, **6**, 1855-1865.
10. Shnyreva, M. G., and Egorov, S. N. (1990) *Mikrobiologiya*, **59**, 948-958.
11. Kelly, R. B. (1985) *Science*, **230**, 25-32.
12. Mueller, S. C., and Branton, D. (1984) *J. Cell Biol.*, **98**, 341-346.
13. Walworth, N. C., and Novick, P. J. (1987) *J. Cell Biol.*, **105**, 163-174.
14. Holcomb, C. L., Etcheverry, T., and Schekman, R. (1987) *Analyt. Biochem.*, **166**, 328-334.
15. Nandi, P. K., Irace, G., van Jaarsveld, P. P., Lippoldt, R. E., and Edelhoch, H. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5881-5885.
16. Mirjuschenko, F. L., Maximova, V. N., and Egorov, S. N. (2002) *Mikrobiologiya*, in press.
17. Semyonova, I. N., Egorov, S. N., and Egorov, N. S. (1986) *Mikrobiologiya*, **55**, 796-799.
18. Torriani, A. (1960) *Biochim. Biophys. Acta*, **38**, 460-479.
19. Horne, R. W. (1965) *Lab. Invest.*, **14**, 1054-1068.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265-275.
21. Panusz, H. T., Graczyka, G., Wilmanska, D., and Scarzynska, D. (1970) *Analyt. Biochem.*, **35**, 404-407.
22. Kaiser, C. A., Gimeno, R. E., and Shaywitz, D. A. (1997) in *Yeast III* (Pringle, J. R., Broach, J. R., and Jones, E. W., eds.) Cold Spring Harbor Laboratory Press, New York, pp. 91-227.
23. Shabalin, Yu. A., and Kulaev, I. S. (1989) *Biokhimiya*, **54**, 68-73.
24. Novick, P., Ferro, S., and Schekman, R. (1981) *Cell*, **25**, 461-469.