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Tight control of the amount of yeast plasma membrane ATPase during changes in growth conditions and gene dosage

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The activity of the plasma membrane ATPase in growing yeast is increased by low pH and by glucose, conditions which result in a higher demand for proton pumping. The amount of enzyme is not significatively modified under these conditions. The amount of ATPase is only slightly increased by introducing extra copies of its gene in autonomous plasmids. In addition, the expression of the ATPase gene in a multi-copy plasmid causes a reduction of the copy number of the plasmid and slows growth. Therefore, overexpression of the ATPase is detrimental for the cell, justifying a regulatory mechanism based on increasing the catalytic activity and not the amount of enzyme.

Plasma membrane; ATPase; Regulation; Overproduction; *(Saccharomyces cerevisiae)*

1. INTRODUCTION

The yeast plasma membrane ATPase is a proton pump [1] essential for cell growth [2,3]. As the activity of the enzyme depends on the growth conditions of the cells [4–6], it was interesting to investigate to what extent changes in the amount of enzyme contribute to the observed changes in activity. In addition, although decreasing the amount of ATPase seriously impairs growth [2,3], the possibility of overproducing the enzyme has not been explored. Our results indicate that the amount of yeast plasma membrane ATPase is tightly controlled, exhibiting only minor changes during phenotypic and genotypic manipulations.

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2. EXPERIMENTAL

2.1. *Strains and growth media*

Saccharomyces cerevisiae strain RS-16 (MATa, leu2-3,112, ura3-251,328,372) was obtained as a segregant of crossing strains GRF18 (MATα, leu2-3,112, his3-11,15, can1, obtained from G.R. Fink) and FL100 (MATa, ura3-251,328,372, obtained from F. Lacroute). Strain BWG1-7A (MATa, ade1-100, his4-519, leu2-3,112, ura3-52, GAL$^+$) was obtained from L. Guarente [7]. Cells were grown on synthetic medium containing 2% glucose or galactose, 0.7% yeast nitrogen base without amino acids (Difco) and uracil (0.2 mM), adenine (0.2 mM), histidine (0.4 mM) and leucine (1 mM) as required. Uracil was omitted for strains transformed with URA3 plasmids.

2.2. *Plasmids*

YCp50 is a pBR322 derivative containing the yeast URA3 gene and a 2.5 kb fragment of yeast

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DNA with origin of replication (ARS1) and centromer (CEN4) [8].

pCGS42 is a pBR322 derivative containing the URA3 gene and a 1.6 kb *Hin*dIII-*Hpa*I fragment with the origin of replication of the yeast 2 μm circle [9]. It was obtained from J. Schaum (Collaborative Research, Waltham, MA). pRS-358 is a YCp50 derivative with a 5 kb *Hin*dIII fragment containing the yeast plasma membrane ATPase gene (PMA1) and flanking sequences [2]. pRS-49 is a pCGS42 derivative containing the same fragment described above. pRS-99 was constructed by ligation of the following three fragments: (i) a 0.75 kb *Eco*RI-*Xho*I fragment containing the GAL1 (galactokinase gene) promoter [3]; (ii) a fragment of 4.2 kb extending from an *Xho*I site 70 bp upstream of the ATPase initiation codon to the *Hin*dIII site downstream of the gene [2,3]; (iii) a 7.1 kb *Eco*RI-*Hin*dIII fragment containing most of plasmid pCGS42. Yeast transformation was by the method of Ito et al. [10].

2.3. *Determination of plasma membrane ATPase activity*

Samples of about 50 mg fresh weight of cells were filtered from the culture medium and immediately dropped into liquid nitrogen. This harvesting procedure results in better preservation of the physiological state of the ATPase than collecting the cells by centrifugation. A crude membrane fraction was prepared by differential centrifugation and the activity of the plasma membrane ATPase determined as described [4]. The assay medium contained 50 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 5.7 with Tris), 5 mM $MgCl_2$, 5 mM NaN_3 (to inhibit mitochondrial ATPase), 0.2 mM ammonium molybdate (to inhibit acid phosphatase) and 50 mM KNO_3 (to inhibit vacuolar ATPase).

2.4. *Quantitative immunoassay for the ATPase*

Samples of about 50 mg fresh weight of cells were harvested by centrifugation, washed with water and homogenized as in [4]. After centrifugation for 10 min at 3000 rpm (Sorvall SS-34 rotor), aliquots of supernatant containing 1 μg protein (quantified by the method of Bradford [11]) were diluted to 200 μl with 0.15 M NaCl and applied to nitrocellulose filters with a Minifold II device (Schleicher & Schuell). This is a filtration manifold with rows of slots suited for quantitative densitometry. Filters were processed as described [12], employing as 1/1000 dilution of anti-ATPase antiserum [2] and ^{125}I-protein A from Amersham. In Western blots performed under similar conditions, the antibody only reacted with the ATPase band of 100 kDa. After autoradiography for 1 h at -70°C with an intensifying screen, the developed film was scanned with a Quick Scan densitometer (Desaga, Heidelberg). Quantification was effected by measuring the height of the peaks and a linear response was observed in the range from 0.2 to 2 μg protein per well.

2.5. *Quantification of mRNA*

Total yeast RNA was prepared as in [13] and quantified by the absorbance at 260 nm. Samples of 400 μl containing 10 μg RNA were denatured by incubation for 15 min at 65°C with 6.15 M formaldehyde and then applied to nitrocellulose filters with the Minifold II device described above. Filters were processed as for Northern analysis [14] with 50% formamide in the hybridization mixture. The probe for the ATPase mRNA was a 0.8 kb *Eco*RI fragment internal to the gene [2] radiolabelled by the technique of Feinberg and Vogelstein [15]. In Northern blots this probe hybridized only with the ATPase mRNA of 3.4 kb. The probe for the URA3 mRNA was the 1.1 kb *Hin*dIII fragment from plasmid YEp24 [16] radiolabelled as above. Autoradiography was performed for 20 min without screen and the developed film scanned as above. A linear response was obtained in the range from 2 to 20 μg RNA per well.

3. RESULTS AND DISCUSSION

Francois et al. [6] have described an increase in plasma membrane ATPase activity during late exponential growth and a decrease at the stationary phase. We have reproduced these observations and demonstrated that the observed changes in activity cannot be explained by regulation of the enzyme level. The levels of ATPase protein and ATPase mRNA are fairly constant during growth and only demonstrate a small decrease at the stationary phase (fig.1). As the level of ATPase is slightly lower (about 20%) in galactose-grown cells than in

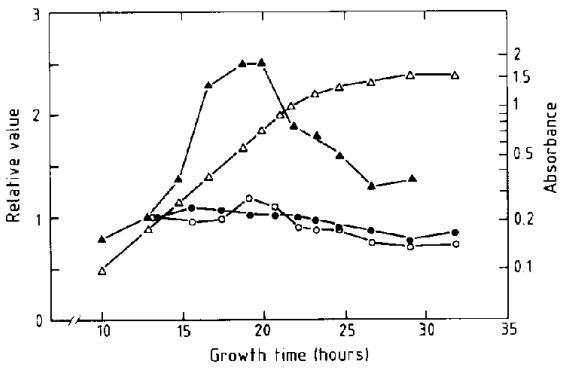

Fig.1. Changes in the plasma membrane ATPase during yeast growth. (△) Absorbance of the culture (660 nm); (▲) relative plasma membrane ATPase activity; (●) relative amount of protein reacting with anti-ATPase antibody; (○) relative amount of ATPase mRNA. Results of a typical experiment are shown. Values are the average of two determinations differing by less than 15%. The values after 14 h of growth are taken as unity.

glucose-grown cells (not shown), the observed decrease may be due to exhaustion of the glucose. These small effects do not preclude considering the ATPase gene as constitutive.

The increase in activity during exponential growth seems to be caused by the progressive acidification of the medium [17]. The ulterior decrease seems a consequence of the exhaustion of glucose [4]. Both regulatory mechanisms may be related because glucose metabolism results in high rates of acid production and external acidity will increase the rate of proton diffusion into the cells. Therefore, a higher demand for proton pumping will result from both glucose and acidification. Both in bacteria [18] and in animal cells [19] the amount of plasma membrane ATPase increases in response to the demand for ion transport (H^+ and Na^+, respectively). Some special features of the yeast system may have precluded this type of regulation. Previous experiments showed that small reductions in the level of yeast plasma membrane ATPase result in drastic impairment of growth [2,3]. We have therefore investigated whether there are similar constraints for increasing the level of enzyme.

A DNA fragment containing the ATPase gene was inserted into either a single-copy, centromeric plasmid (YCp50) or a multi-copy, 2u-derived plasmid (pCGS42). When these constructions were introduced into yeast only modest increases in ATPase were observed (20 and 50%, table 1). A higher increase was observed for the ATPase mRNA (60 and 220%), suggesting that controls at the level of protein synthesis and/or degradation prevent the ATPase from increasing proportionally to its mRNA. In addition, the observed increase in ATPase mRNA in the multi-copy plasmid was much lower than expected. The 2u-derived plasmids used have copy numbers of 10–30 per cell [9] and the activity of some enzymes cloned into these plasmids is elevated 7–14-fold [20]. We have therefore investigated the level of URA3 mRNA as an indirect estimation of the copy number of the plasmids. This gene codes for the soluble, biosynthetic enzyme orotidine-5′-phosphate carboxylyase [21] and would not be subjected to the regulatory constraints of the ATPase. As indicated in table 1, when the URA3 gene is present alone in the multi-copy plasmid the corresponding mRNA is increased about 13-fold, suggesting a high copy number for the plasmid. On the other hand, when both the URA3 and PMA1 (ATPase) genes are present in the same plasmid the URA3 mRNA is only increased about 4-fold. Therefore, the ATPase gene probably causes a reduction in the copy number of the plasmid, suggesting that it is deleterious for the cell. Accordingly, the growth rate is slightly decreased (about 20%) in the ATPase-overproducing strain.

In order to investigate further this phenomenon we have placed the ATPase gene in the multi-copy plasmid under galactose control. This was effected by replacing the constitutive promoter of the ATPase gene by the GAL1 promoter. In galactose medium, where the ATPase gene in the plasmid is expressed, the levels of ATPase and URA3 mRNA are only 2-fold greater than in the control strain, suggesting a low copy number for the plasmid (table 2). With a plasmid containing only the URA3 gene the level of URA3 mRNA is increased 10-fold, suggesting a high copy number. On the other hand, in glucose medium, where the ATPase gene in the plasmid is not expressed, the level of URA3 mRNA is only slightly reduced by the presence of the ATPase gene on the plasmid (from 6- to 4-times over the control, table 2). This indicates that it is the expression of the ATPase gene and not merely its presence in the plasmid that

Table 1

Effect of increasing gene dosage with plasmids on the level of ATPase

Plasmid	Growth rate (h^{-1})	Plasma membrane ATPase		URA3 mRNA
		Protein	mRNA	
Single-copy				
YCp50 (URA3)	0.28	"1"	"1"	"2"
pRS-358 (URA3, PMA1)	0.28	1.2	1.6	–
Multi-copy				
pCGS42 (URA3)	0.28	1.0	1.0	13.4
pRS-49 (URA3, PMA1)	0.23	1.5	3.2	4.4

Strain RS-16 was transformed with the indicated plasmids, grown on glucose medium and harvested during the exponential phase (absorbance at 660 nm of 0.3–0.4). Results are the average of two determinations differing by less than 10%. Values between quotation marks are the reference for the relative values of every column. The URA3 gene is present in two copies in the reference strain (one in the chromosome and another in the plasmid) and therefore a value of "2" was assigned for the relative level of URA3 mRNA

causes the apparent reduction in copy number.

The copy control system for the yeast 2u plasmids is based on an amplification mechanism, which increases the copy number in daughter cells receiving fewer copies than the mother cell [9]. This means that during growth copy number changes continuously and, if this results in differences in growth rate, selection may occur. Overproduction of the ATPase seems to slow growth (tables 1,2) and this will result in selection of cells with low copy number. The reason for the toxicity of overexpressing the ATPase is not known. The enzyme is a major component of the plasma membrane, accounting for 15–20% of the plasma membrane protein [1]. Therefore, there may be structural constraints for increasing the amount of

Table 2

Effect of increasing the dosage of an ATPase gene under galactose control

Plasmid	Growth rate (h^{-1})	Plasma membrane ATPase		URA3 mRNA
		Protein	mRNA	
Expt 1: galactose medium				
None	0.14	"1"	"1"	"1"
pCGS42 (URA3)	0.14	1.0	0.9	10.0
pRS-99 (URA3, PMA1)	0.10	1.5	2.1	2.0
Expt 2: glucose medium				
None	0.20	"1"	"1"	"1"
pCGS42 (URA3)	0.20	1.0	1.0	6.0
pRS-99 (URA3, PMA1)	0.20	1.0	1.1	4.0

Strain BWG1-7A and its transformant derivatives were grown on either galactose or glucose media. Other conditions as in table 1

enzyme without affecting the integrity of the membrane. Other less abundant plasma membrane proteins, such as the uracil [22] and purine-cytosine [23] permeases can be easily overproduced with multi-copy plasmids. On the other hand, the plasma membrane ATPase is a major ATP consumer [24] and its overproduction may compromise the energy charge of the cell. In any case, the limitations in modifying the amount of ATPase indicated by these and previous [2,3] experiments justify that in the case of yeast a change in the demand for ion transport is coped with by modulating the catalytic activity and not the amount of enzyme.

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REFERENCES

[1] Serrano, R. (1985) Plasma Membrane ATPase of Plants and Fungi, CRC Press, Boca Raton, FL.
[2] Serrano, R., Kielland-Brandt, M.C. and Fink, G.R. (1986) Nature 319, 689–693.
[3] Cid, A., Perona, R. and Serrano, R. (1987) Curr. Genet. 12, 105–110.
[4] Serrano, R. (1983) FEBS Lett. 156, 11–14.
[5] Tuduri, P., Nso, E., Dufour, J.P. and Goffeau, A. (1985) Biochem. Biophys. Res. Commun. 133, 917–922.
[6] Francois, J., Eraso, P. and Gancedo, C. (1987) Eur. J. Biochem. 164, 369–373.
[7] Guarente, L., Yocum, R.R. and Gifford, P. (1982) Proc. Natl. Acad. Sci. USA 79, 7410–7414.
[8] Johnston, M.J. and Davis, R.W. (1984) Mol. Cell. Biol. 4, 1440–1448.
[9] Broach, J.R. (1983) Methods Enzymol. 101, 307–325.
[10] Ito, H., Fukuda, Y. and Kimura, A. (1983) J. Bacteriol. 153, 163–168.
[11] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[12] Jahn, R., Schiebler, W. and Greengard, P. (1984) Proc. Natl. Acad. Sci. USA 81, 1684–1687.
[13] Carlson, M. and Botstein, D. (1982) Cell 28, 145–154.
[14] Anderson, M.L.M. and Young, B.D. (1985) in: Nucleic Acid Hybridisation: a Practical Approach (Hames, B.D. and Higgins, S.J. eds) pp.73–111, IRL Press, Oxford.
[15] Feinberg, A.P. and Vogelstein (1983) Anal. Biochem. 132, 6–13.
[16] Botstein, D., Falco, S.C., Stewart, S.E., Brennan, M., Scherer, S., Stinchcomb, D.T., Struhl, K. and Davis, R.W. (1979) Gene 8, 17–24.
[17] Eraso, P. and Gancedo, C. (1987) FEBS Lett. 224, 187–192.
[18] Kobayashi, H., Suzuki, T. and Unemoto, T. (1986) J. Biol. Chem. 261, 627–630.
[19] Wolitzky, B.A. and Fambrough, D.M. (1986) J. Biol. Chem. 261, 9990–9999.
[20] Rine, J., Hansen, W., Hardeman, E. and Davis, R.W. (1983) Proc. Natl. Acad. Sci. USA 80, 6750–6754.
[21] Rose, M., Grisafi, P. and Botstein, D. (1984) Gene 29, 113–124.
[22] Chevallier, M.R. (1984) Mol. Cell. Biol. 2, 977–984.
[23] Schmidt, R., Manolson, M.F. and Chevallier, M.R. (1984) Proc. Natl. Acad. Sci. USA 81, 6276–6280.
[24] Serrano, R. (1980) Eur. J. Biochem. 105, 419–424.