FEBS Letters 453 (1999) 210–214 FEBS 22176

Enlargement of the endoplasmic reticulum membrane in *Saccharomyces cerevisiae* is not necessarily linked to the unfolded protein response via Ire1p

An K. Stroobants, Ewald H. Hettema, Marlene van den Berg, Henk F. Tabak*

Department of Biochemistry, Academic Medical Centre, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

Received 30 March 1999

Abstract Conditions that stress the endoplasmic reticulum (ER) in *Saccharomyces cerevisiae* can elicit a combination of an unfolded protein response (UPR) and an inositol response (IR). This results in increased synthesis of ER protein-folding factors and of enzymes participating in phospholipid biosynthesis. It was suggested that in cells grown on glucose or galactose medium, the UPR and the IR are linked and controlled by the ER stress sensor Ire1p. However, our studies suggest that during growth on oleate the IR is controlled both by an Ire1p-dependent pathway and by an Ire1p-independent pathway.

Key words: Unfolded protein response; Karmella; Peroxisome biogenesis; *INO1*

1. Introduction

Saccharomyces cerevisiae is well equipped to cope with stressful conditions arising from the surroundings, such as heat shock, osmotic or oxidative stress [1]. However, internal conditions can also change and cause difficulties that a yeast cell must try to surmount in order to prolong its life. This is, for instance, the case when proteins accumulate in the endoplasmic reticulum (ER). Proteins of the endosomal compartment or proteins to be secreted are folded in the ER and sometimes oligomerized into multi-protein complexes. A number of folding factors collectively called chaperones assist in these processes, for instance Kar2p, Pdi1p, FKB2 and Eug1p [2]. The success of folding and oligomerization is controlled in the ER by a 'quality control system'. When a protein fails the test, it is not allowed to continue its route to the Golgi apparatus and subsequent compartments. Rather, it remains in the ER for a longer time to complete folding, or it is eventually degraded [3].

In response to the accumulation of proteins, the volume of the ER increases. This is achieved by induction of the unfolded protein response (UPR), which results in enhanced synthesis of additional chaperones and in the inositol response (IR), which stimulates synthesis of membrane lipids. The UPR pathway has been carefully dissected and its components have been characterized [4]. Here we mention two components.

A pivotal role is played by Ire1p [5], an integral membrane protein of the ER and the perinuclear membrane. Its N-terminal half, residing in the ER lumen, detects accumulation of proteins while the C-terminal half, reaching out into the nucleus, has a dual function. Ire1p is a protein kinase which undergoes autophosphorylation upon oligomerization; this is thought to activate its ribonuclease activity which initiates splicing of a precursor mRNA encoding Hac1p.

Hac1p is a transcription factor and the second component of the UPR [6–8]. It activates transcription of genes that share an unfolded protein response element (UPRE) in their promoters. Most of these genes encode chaperones and thus close the UPR regulatory loop. It was argued that the increase in enzymes required for enhanced phospholipid biosynthesis (via the IR) is also mediated by Ire1p and Hac1p [9]. In this view, UPR and IR are bifurcations from the same stress sensor, i.e. Ire1p (Fig. 1).

The importance to the yeast cell of this combined response to intracellular stress is exemplified by the behavior of an *ire1* mutant. When cells were stressed by incubation with either tunicamycin (an inhibitor of *N*-linked glycosylation) or reducing agents (interfering with protein folding) Δ*ire1* cells died [10]. Furthermore, Δ*ire1* cells proved to be inositol auxotrophs [5], suggesting that the IR is compromised in the *ire1* mutant. Overexpression of the ER integral membrane protein HMG-CoA reductase 1 (Hmg1p) normally results in an increase in the ER compartment and in accumulation of Hmg1p in stacked ER membranes called karmellae [11,12]. Interestingly, Δ*ire1* cells died upon overexpression of Hmg1p, probably because they were unable to induce both a UPR and an IR [9].

We tried to use this information in our studies on the biogenesis and function of peroxisomes. In one of our genetic screens we discovered *PEX15* [13]. *PEX15* encodes an integral peroxisomal membrane protein and loss of its function results in mislocalization of peroxisomal matrix proteins to the cytoplasm and loss of the typical ovoid-shaped peroxisomal morphology as revealed by electron microscopy. Several observations suggested routing of Pex15p to peroxisomes via the ER. Expressed pex15p (or Pex15p derivatives) accumulated in ER-like membranes, which were continuous with the perinuclear membrane. Furthermore, a fusion protein of Pex15p and invertase lacking its ER targeting signal was glycosylated and the stacked membranes resembled karmellae [14]. Other groups also made observations fuelling the idea that the ER might be involved in some aspects of peroxisome biogenesis. For instance, mutants in *Yarrowia lipolytica* that were defective in the secretion of proteins via ER and Golgi apparatus, also proved to be disturbed in the biogenesis of peroxisomes [15]. In addition, two peroxisomal integral membrane proteins were shown to be targeted to the ER and to be glycosylated

*Corresponding author. Fax: (31) (20) 6915519.
E-mail: h.f.tabak@amc.uva.nl

Abbreviations: Hmg1p, hydroxy-methyl-glutaryl-CoA reductase isoenzyme 1; GFP, green fluorescent protein; UAS, upstream activating sequence

PII: S0014-5793(99)00721-8

before they reached the peroxisome [16]. Treatment of *Hansenula polymorpha* with brefeldin A, an inhibitor of ER/Golgi vesicular traffic, resulted in accumulation of peroxisomal matrix and membrane proteins in an ER-like compartment [17].

To further try to identify the ER as a possible link in the chain of events leading to the formation of peroxisomes, we wondered whether accumulation of Pex15p would be deleterious to a cell that cannot induce a UPR due to deletion of IRE1. Indeed, the *ire1* mutant died when Pex15p was produced at elevated levels. However, when we overproduced a mitochondrial integral membrane protein as a control, to our surprise *ire1* cells also died, which questions the specificity of the UPR and IR. Peroxisomes increase in volume and number when yeast grows on a fatty acid as sole carbon source [18]. When we repeated the expression of membrane proteins with *ire1* cells and grew the cells on oleate instead of glucose or galactose, these did not die and karmella-like membranes were still formed. In addition, cells grown on oleate were not dependent on external inositol, in contrast to *ire1* cells grown on glucose or galactose. Here, we discuss some implications of our findings.

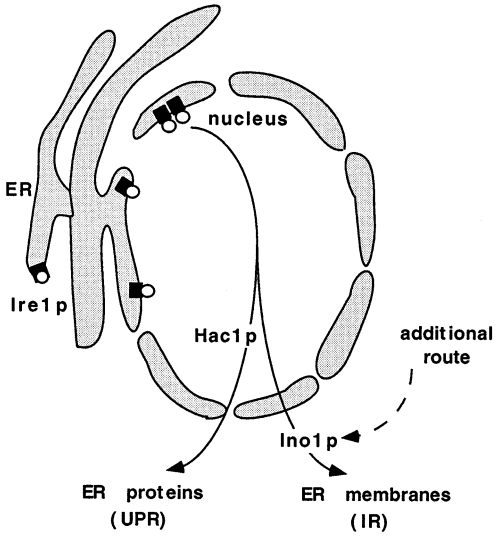

Fig. 1. Schematic representation of Ire1p signalling, leading to the synthesis of ER proteins via the UPR as well as the synthesis of ER membranes via the IR.

2. Materials and methods

The *S. cerevisiae* strains used were: 'wild type' JC104 and 'Δire1' CS173 [10] (a kind gift from R. Chapman); 'wild type' BJ1991 (Matα; *leu2, trp1, ura3-251, prb1-1122, pep4-3*) and 'Δire1' (the same as BJ1991 except *ire1*::URA3), generated with disruption plasmid PCS135 [10], kindly provided by R. Chapman). Δ*pip2*, Δ*oaf1* and Δ*pip2*/Δ*oaf1* were also made in BJ1991, as described in [19]. Synthetic minimal media with or without inositol were used as described by Sherman [20]. As carbon source we used either 0.3% or 2% (w/v) glucose, or 2% (w/v) galactose, or 0.1% (w/v) oleate/0.4% (w/v) Tween-40.

The plasmids used were based on YEplac112 [21], with either the gal1/10 or the catalase promoter [22] cloned between the *Eco*RI and *Sac*I sites, with the NH tag in *Sac*I-*Bam*HI and with the *PEX15*, *ACR1* or *PEX14* ORFs in *Bam*HI-*Hin*dIII. The Hmg1p expression constructs were made by cloning a *HMG1* fragment that was fused in-frame to *GFP* from plasmid pCR425 (a kind gift from R. Wright) and cut with *Bam*HI and *Xho*I into the YEplac112 vector containing the gal1/10 promoter or the catalase promoter cut with *Bam*HI and *Sal*I. The inv-GFP-HDEL construct used was described in [23].

ORFs and promoter sequences were amplified by PCR using specific primers introducing restriction sites directly before the translation-initiation site or directly after the stop codon. The *INO1* ORE fragment was obtained by annealing oligonucleotides F-ORE AATTCAGTGATCGGAACGAGCTCTTTATCACCGTAG and R-ORE GATCCTACGGTGATAAAGAGCTCGTTCCGATCACTG; 1 pmol of each in 1×SSC was slowly cooled from 100°C to room temperature. The *FOX3* ORE used was described in [24].

Techniques used were: DNA manipulations [25], yeast transformation [26] and bandshift assays (extract preparations, labelling and analysis) [19]. SAGE data were obtained from [27]. Immunolabelling of ultra-thin cryo-sections with polyclonal antibodies against NH ([22], a kind gift from P. van der Sluijs) and against GFP (Clontech) was performed according to [28].

3. Results

3.1. Pex15p induced karmella formation

Northern blot and SAGE analysis indicated that *PEX15* and other *PEX* genes were expressed at low steady-state levels (less than 1 mRNA copy per cell), more or less irrespective of the carbon source in the medium [27]. Expression of *PEX15* from an expression plasmid in cells grown on oleate induced karmella-like structures which contained Pex15p as shown before (Fig. 2A).

In order to test whether these membranes were ER membranes, we developed a GFP derivative as ER marker. The presequence of invertase was appended to the N-terminus of *GFP* to function as an ER-addressing signal and the yeast ER retention signal (HDEL) was fused to the C-terminal end of *GFP* [23]. Expression of this fusion protein (pre*INV-GFP*-HDEL) and inspection of the cells by immuno-gold electron microscopy showed membranes decorated with gold particles that had all the characteristics of the ER compartment: staining of the perinuclear membrane and membranes located at the periphery of the cell close to the plasma membrane (Fig. 2B). Co-expression of Pex15p (small gold particles) and pre-*INV-GFP*-HDEL (large gold particles) showed exact co-localization of small and large gold particles, proving that under certain conditions Pex15p is indeed associated with the ER (Fig. 2B–D).

3.2. Expression of membrane proteins in Δire1 grown on galactose or oleate

Based on these findings, we hypothesized that yeast cells start the UPR/IR upon Pex15p overexpression and we expected that an *ire1* mutant would die under these conditions. To test this hypothesis, we expressed Pex15p under control of the gal1/10 promoter (the experimental set-up of Cox et al. [9]). Indeed, the Δ*ire1* transformants died (Fig. 3A). Although we initially interpreted this result as an additional suggestion for a possible functional relationship between peroxisomes and the ER, a number of disturbing observations were made when we carried out additional controls.

A protein that has been used in studies on karmella formation and the UPR is Hmg1p. When we studied the UPR in cells grown on oleate-containing medium and used Hmg1p expressed from the catalase promoter to stress the ER, to our surprise both wild type and *ire1* mutant cells showed formation of karmella-like membrane structures (Fig. 2E,F) and *ire1* cells did not die despite the compromised UPR/IR pathways (compare Fig. 3A with Fig. 3B). The same result was obtained when the integral peroxisomal membrane protein Pex15p was expressed on oleate in *ire1* cells (Fig. 3B).

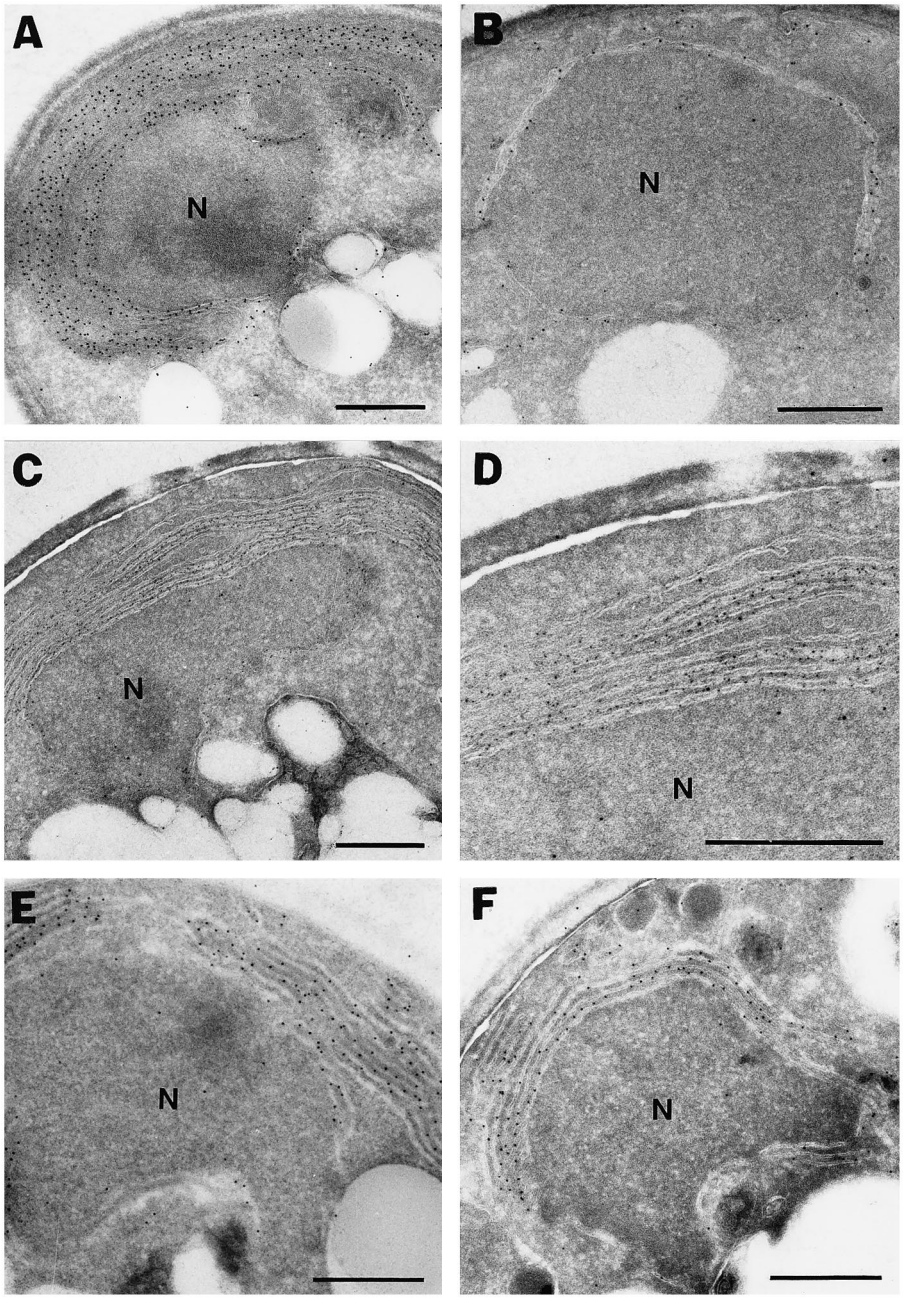

Fig. 2. Electron microscopic analysis of the location of Pex15 in karmellae in oleate-grown wild type cells transformed with: (A) NH-*PEX15*/2μ; (B) inv-*GFP*-HDEL/CEN; (C) NH-*PEX15*/2μ and inv-*GFP*-HDEL/CEN; (D) detail of C. Analysis of the formation of karmellae in oleate-grown wild-type cells (E) and Δ*ire1* cells (F), transformed with *HMG1-GFP*/2μ. Antibodies used were α-NH (A,C,D) and α-GFP (B,C,D,E,F). N = nucleus. Bar = 0.2 μm.

Integral membrane proteins from other organelles were expressed at elevated levels as controls. A typical example shown here is Acr1p, an integral membrane protein of the mitochondrial inner membrane [29]. Contrary to expectation, *ire1* cells died on galactose-containing medium due to Acr1p expression (Fig. 3A). Wild type cells expressing Acr1p do not show formation of karmellae and immuno-gold labelling indicated that Acr1p was only present in mitochondria. Western blot analysis indicated that the proteins (tagged versions of Hmg1p, Pex15p and Acr1p) were expressed at similar levels (not shown). On the other hand, production of the peripheral peroxisomal membrane protein Pex14p did not compromise the *ire1* mutant (when grown on galactose, not shown). Again, *ire1* cells expressing Acr1p survived on oleate-containing medium (Fig. 3B). It is clear that the aspecificity of the UPR/IR that we observed in our experiments does not allow us to draw conclusions with regard to a functional ER-to-peroxisome relationship. Despite this, we tried to explain the discrepancy between the phenotypes of *ire1* cells grown on media containing different carbon sources.

3.3. Regulation of *INO1* expression

Yeast cells with a non-functional Ire1p display auxotrophy to inositol [30]. Surprisingly, in our experiments addition of

inositol to oleate-containing minimal plates was not necessary to allow growth of *ire1* cells. Inositol is an essential precursor for the synthesis of phosphatidylinositols and is an important controller of phospholipid biosynthesis. It is produced from glucose 6-phosphate by the enzyme Ino1p [30]. Both the *INO1* gene and the enzyme are caught in a delicate network of control. Well-known components of the transcriptional control circuit comprise Opi1p, a factor repressing transcription of the *INO1* gene, and Ino2p and Ino4p, both promoting its transcription [30–32]. However, additional components may be involved. In our studies on peroxisome biogenesis using genome-wide transcription analysis with SAGE, we noticed that in cells grown on oleate the *INO1* mRNA level was much higher than in a mutant lacking two important transcription factors (pip2 and oaf1) that are required for induction of genes coding for peroxisomal proteins (21 copies per cell versus one)[27]. The Pip2p/Oaf1p heterodimer binds to a *cis*-acting DNA sequence called oleate response element (ORE) that is present in the promoters of such genes [19,33,34]. Interestingly, the *INO1* gene contained a putative ORE at position −368 to −347. To test whether this is a functional ORE, we carried out a DNA bandshift experiment. A DNA segment of 36 bp containing the *INO1* 'ORE' was radiolabelled and incubated with protein extracts of wild type and mutant cells (Δ*pip2*, Δ*oaf1* and the double mutant Δ*pip2*/Δ*oaf1*) grown on either glucose or oleate as carbon source. ORE DNA from the *FOX3* promoter served as a control (Fig. 4). The *INO1* ORE fragment gave rise to a specific retardation complex similar to that obtained with *FOX3* DNA. It was absent when *INO1* DNA (or *FOX3* DNA) was incubated with the mutant extracts, indicating that Pip2p and Oaf1p are responsible for the formation of this retarded complex.

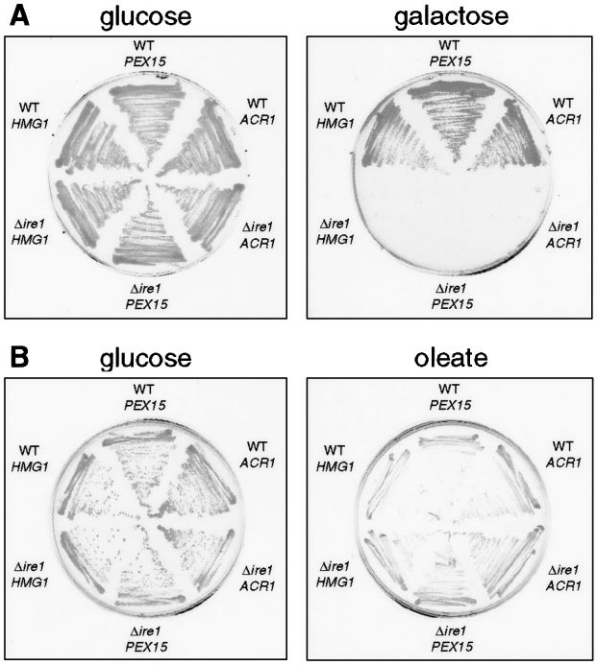

Fig. 3. Growth of wild type and Δ*ire1* cells transformed with expression constructs encoding integral membrane proteins under control of the galactose-inducible gal1/10 promoter (A) or oleate-inducible catalase promoter (B). Cells were transformed with *HMG1-GFP*/2μ, NH-*PEX15*/2μ or NH-*ACR1*/2μ. Growth is shown on selective glucose before induction (A+B) and selective galactose (A) or selective oleate (B) after induction.

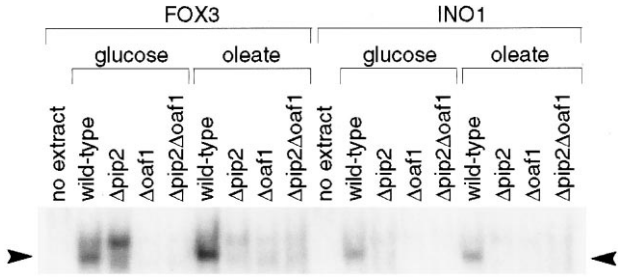

Fig. 4. DNA bandshift experiment, showing binding of radiolabelled ORE DNA fragments of *FOX3* (control) and *INO1* to a protein complex (arrowheads) which is present in wild type cells and absent in Δ*pip2* and Δ*oaf1* cells. Protein extracts were obtained from glucose- (0.3%) and oleate-grown cultures.

Although this suggests that the *INO1* gene contains a functional ORE and could thus explain that *INO1* is actively transcribed in cells grown on oleate, we have not yet been able to show that the *INO1* ORE can activate a reporter gene on its own, as we reported for the ORE of the *FOX3* gene [24].

4. Discussion

We showed that in *S. cerevisiae*, overproduction of the peroxisomal integral membrane protein Pex15p resulted in karmella formation and cell death in an *ire1* genetic background, just like by overproduction of the integral ER membrane protein Hmg1p, when using a galactose induction system. Two explanations may be considered to understand this observation. (i) Pex15p first targets to the ER before arriving in the peroxisomal membrane. This would be in line with recent suggestions that the ER could be involved in the biogenesis of peroxisomes. (ii) The ER membranes readily take up hydrophobic proteins that do not reach their normal acceptor membrane in time. In that case the fact that an overproduced peroxisomal membrane protein was found in ER membranes does not provide an argument for a functional relationship between ER and peroxisomes. We favor the second possibility, based on the fact that in Δ*ire1* production of the inner mitochondrial membrane protein Acr1p from an expression plasmid resulted in cell death too.

S. cerevisiae with an *ire1* mutation was dependent on the addition of inositol to a minimal glucose-based or galactose-based growth medium. The intracellular concentration of inositol functions as an important sensor to control the biosynthesis of phospholipids, both at the enzymatic level and at the transcriptional level [30]. The combined loss of the UPR (ire1) and inositol prototrophy (IR) was worked out in further detail to support a model in which Ire1p and Hac1p coordinate the production of ER proteins as well as of enzymes involved in the synthesis of ER phospholipids [9]. Although such a coordination might be the case under certain conditions of growth in which either glucose or galactose is provided as carbon source, our experiments show that in cells grown on oleate this link was not apparent. Δ*ire1* cells grew perfectly well in minimal medium with oleate as sole carbon source without addition of inositol. Remarkably, we still observed enlargement of the ER compartment (karmella formation) upon overexpression of Pex15p or Hmg1p. In fact, electron microscopy revealed initial karmella formation when Δ*ire1* cells were shifted to galactose medium to induce Pex15p or Hmg1p expression (results not shown).

Taken together, our results suggest that the IR is not necessarily linked to the UPR and that it can be independently controlled in additional ways (Fig. 1). This is in line with another study in which *IRE1* gene disruption did not prevent cytochrome P450-induced ER proliferation [35].

In a genome-wide analysis of transcription using serial analysis of gene expression (SAGE), we observed that *INO1* mRNA is present at a relatively high copy number per cell (21) in cells grown on oleate. This would explain why an *ire1* mutant displayed no inositol auxotrophy in a growth medium containing fatty acids. Furthermore, the *INO1* mRNA level dropped in the *pip2/oaf1* double mutant lacking the transcription factors required for induction of genes coding for peroxisomal proteins in cells grown on oleate. Preliminary experiments on the *INO1* promoter suggest that it indeed contains an ORE, to which Pip2p/Oaf1p can bind. This would be in addition to the UAS_{ino} served by the Ino2p/Ino4p factors, another positive mode of control of the expression of the *INO1* gene. The expression of the *INO1* gene should, we feel, be studied in more detail, since its product plays such an important role in the coordination of phospholipid biosynthesis in the cell.

Acknowledgements: We thank Rowan Chapman for helpful discussions and strains, Robin Wright for gifts of plasmids, and Peter van der Sluijs for NH antibodies. This work was financially supported by the Netherlands Foundation for Scientific Research NWO-MW.

References

[1] Hohmann, S. and Mager, W.H. (1997) Yeast Stress Responses, R.G. Landes, Austin, TX.
[2] Shamu, C.E., Cox, J.S. and Walter, P. (1994) Trends Cell Biol. 4, 56–60.
[3] Hammond, C. and Helenius, A. (1995) Curr. Opin. Cell Biol. 7, 523–529.
[4] Sidrauski, C., Chapman, R. and Walter, P. (1998) Trends Cell Biol. 8, 245–249.
[5] Nikawa, J. and Yamashita, S. (1992) Mol. Microbiol. 6, 1441–1446.
[6] Shamu, C.E. (1998) Curr. Biol. 8, 121–123.
[7] Cox, J.S. and Walter, P. (1996) Cell 87, 391–404.
[8] Kawahara, T., Yanagi, H., Yura, T. and Mori, K. (1997) Mol. Biol. Cell 8, 1845–1862.
[9] Cox, J.S., Chapman, R.E. and Walter, P. (1997) Mol. Biol. Cell 8, 1805–1814.
[10] Cox, J.S., Shamu, C.E. and Walter, P. (1993) Cell 73, 1197–1206.
[11] Parrish, M.L., Sengstag, C., Rine, J.D. and Wright, R.L. (1995) Mol. Biol. Cell 6, 1535–1547.
[12] Wright, R., Basson, M., D'Ari, L. and Rine, J. (1988) J. Cell Biol. 107, 101–114.
[13] Elgersma, Y., Van den Berg, M., Tabak, H.F. and Distel, B. (1993) Genetics 135, 731–740.
[14] Elgersma, Y., Kwast, L., van den Berg, M., Snyder, W.B., Distel, B., Subramani, S. and Tabak, H.F. (1997) EMBO J. 16, 7326–7341.
[15] Titorenko, V.I., Ogryziak, D.M. and Rachubinski, R.A. (1998) Mol. Cell. Biol. 17, 5210–5226.
[16] Titorenko, V.I. and Rachubinski, R.A. (1998) Mol. Cell. Biol. 18, 2789–2803.
[17] Salomons, F.A., van der Klei, I.J., Kram, A.M., Harder, W. and Veenhuis, M. (1997) FEBS Lett. 411, 133–139.
[18] Veenhuis, M., Mateblowski, M., Kunau, W.H. and Harder, W. (1987) Yeast 3, 77–84.
[19] Rottensteiner, H., Kal, A.J., Binder, M., Hamilton, B., Tabak, H.F. and Ruis, H. (1996) EMBO J. 15, 2924–2934.
[20] Sherman, F. (1991) Methods Enzymol. 194, 3–21.
[21] Gietz, R.D. and Sugino, A. (1988) Gene 74, 527–534.
[22] Elgersma, Y. (1995) Transport of Proteins and Metabolites Across the Peroxisomal Membrane in *Saccharomyces cerevisiae*, Thesis, University of Amsterdam.
[23] Hettema, E.H., Ruigrok, C.C.M., Groot Koerkamp, M., van den Berg, M., Tabak, H.F., Distel, B. and Braakman, I. (1998) J. Cell Biol. 27, 421–434.
[24] Einerhand, A.W.C., Voorn-Brouwer, T.M., Erdmann, R., Kunau, W.H. and Tabak, H.F. (1991) Eur. J. Biochem. 200, 113–122.
[25] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[26] Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) J. Bacteriol. 153, 163–168.
[27] Kal, A.J., van Zonneveld, A.J., Benes, V., van den Berg, M., Groot Koerkamp, M.G., Albermann, K., Strack, N., Ruijter, J.M., Richter, A., Dujon, B., Ansorge, W. and Tabak, H.F. (1999) Mol. Biol. Cell (in press).
[28] Gould, S.J., Keller, G.A., Schneider, M., Howell, S.H., Garrard, L.J., Goodman, J.M., Distel, B., Tabak, H.F. and Subramani, S. (1990) EMBO J. 9, 85–90.
[29] Verleur, N. (1998) Permeability Properties of Peroxisomes, Thesis, University of Amsterdam.
[30] Greenberg, M.L. and Lopes, J.M. (1996) Microbiol. Rev. 60, 1–20.
[31] Nikoloff, D.M., McGraw, P. and Henry, S.A. (1992) Nucleic Acids Res. 20, 3253.
[32] Klig, L.S., Hoshizaki, D.K. and Henry, S.A. (1988) Curr. Genet. 13, 7–14.
[33] Einerhand, A.W.C., Kos, W.T., Distel, B. and Tabak, H.F. (1993) Eur. J. Biochem. 214, 323–331.
[34] Luo, Y., Karpichev, I.V., Kohanski, R. and Small, G.M. (1996) J. Biol. Chem. 271, 12068–12075.
[35] Menzel, R., Vogel, F., Kargel, E. and Schunck, W.H. (1997) Yeast 13, 1211–1229.