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Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter

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In response to heavy metal stress, plants and certain fungi, such as the fission yeast *Schizosaccharomyces pombe*, synthesize small metal-binding peptides known as phytochelatins. We have identified a cadmium sensitive *S.pombe* mutant deficient in the accumulation of a sulfide-containing phytochelatin–cadmium complex, and have isolated the gene, designated *hmt1*, that complements this mutant. The deduced protein sequence of the *hmt1* gene product shares sequence identity with the family of ABC (ATP-binding cassette)-type transport proteins which includes the mammalian P-glycoproteins and CFTR, suggesting that the encoded product is an integral membrane protein. Analysis of fractionated fission yeast cell components indicates that the HMT1 polypeptide is associated with the vacuolar membrane. Additionally, fission yeast strains harboring an *hmt1*-expressing multicopy plasmid exhibit enhanced metal tolerance along with a higher intracellular level of cadmium, implying a relationship between HMT1 mediated transport and compartmentalization of heavy metals. This suggests that tissue-specific overproduction of a functional *hmt1* product in transgenic plants might be a means to alter the tissue localization of these elements, such as for sequestering heavy metals away from consumable parts of crop plants.

Key words: cadmium resistance/CdS crystallites/metal-binding peptides/phytochelatins/*Schizosaccharomyces pombe*

Introduction

Heavy metals such as Cu and Zn perform vital structural and functional roles in cell metabolism. In excess, however, these as well as non-essential metals, e.g. Cd, Hg and Pb, can be detrimental to cellular processes. Numerous human and animal disorders are attributed to environmental contamination by heavy metals and a number of studies also suggest that exposure to high Cd levels is associated with a heightened risk of cancer (Heinrich, 1988). Chelation can ameliorate heavy metal toxicity by effectively lowering the intracellular concentration of the free metal ions. Animals and certain fungi, such as *Saccharomyces cerevisiae* and *Neurospora crassa*, induce the synthesis of metallothioneins, cysteine-rich proteins that bind heavy metals via formation of thiolate bonds (Hamer, 1986). Plants and other fungi, such as *Candida glabrata* and the fission yeast *Schizosaccharomyces pombe*, synthesize small peptides that are not products of RNA translation. Derived from glutathione (GSH; γ-Glu-Cys-Gly), they have the general structure $(\gamma\text{-Glu-Cys})_n\text{Gly}$, where n is from 2 to 11 (Grill *et al.*, 1985) and have been referred to in the literature by a variety of names: cadystins (Murasugi *et al.*, 1981), phytochelatins (Grill *et al.*, 1985), γ-glutamyl peptides (Reese and Winge, 1988) or Cd-binding peptides (Vogeli-Lange and Wagner, 1990). As in a recent review (Rauser, 1990), we will refer to them as phytochelatins (PCs).

Synthesis of PCs from GSH is catalyzed by PC synthase, an enzyme present in plant cells regardless of prior exposure to heavy metals (Grill *et al.*, 1989; Löffler *et al.*, 1989). Studies *in vitro* with the purified enzyme indicate that heavy metals activate PC synthase to initiate PC synthesis. The PCs so produced eventually deactivate enzyme activity by chelating the heavy metal cofactors. Recently, Hayashi *et al.* (1991) reported that fission yeast cell-free extracts can also polymerize γ-Glu-Cys and GSH to form $(\gamma\text{-Glu-Cys})_n$ *in vitro*. A Gly terminal residue is then added by glutathione synthetase to form PCs. It is not clear, however, whether this is a significant alternative pathway of PC synthesis *in vivo*.

The relevance of PCs to heavy metal tolerance is substantiated by the observation that fission yeast mutants defective in PC synthesis are more sensitive to heavy metals than wild type strains (Mutoh and Hayashi, 1988). Similar plant mutants have not yet been identified; however, treatment of *Lycopersicum esculentum* and *Rauvolfia serpentina* suspension cell cultures with buthionine sulfoximine, a specific inhibitor of glutathione synthesis, diminishes PC production and impairs growth on Cd-containing media (Steffens *et al.*, 1986). Aside from heavy metal detoxification, it has been proposed that PCs also play a role in metal homeostasis (Rauser, 1990). PC–Cu and PC–Zn complexes have been shown to be capable of efficiently activating metal-depleted apoenzymes *in vitro* (Thumann *et al.*, 1991). This raises the possibility that PC–metal complexes could be a cytoplasmic source of metal cofactors.

Two different forms of PC–metal complexes are found in fission yeast cells exposed to Cd (Murasugi *et al.*, 1983). One species, composed mostly of PCs and Cd, elutes from gel filtration columns with an apparent molecular weight of 3–4 kDa (LMW PC–Cd complex). A second, more highly charged form has an apparent molecular weight of 6–9 kDa, and contains sulfide in addition to PCs and Cd (HMW PC–Cd–S^{-2} complex). The addition of S^{-2} imparts a higher Cd-binding capacity and enhanced stability to the PC–Cd complex (Reese and Winge, 1988). *S.pombe* mutants defective in production of the HMW form are more

sensitive to Cd than wild type strains (Mutoh and Hayashi, 1988), suggesting that production of this complex is essential for heavy metal detoxification. This HMW complex also exhibits some unique structural features. Biophysical analysis suggests that it consists of a CdS crystallite core with quantum semiconductor characteristics, and an outer coat of PC peptides (Dameron *et al.*, 1989).

In this paper, we describe the isolation and characterization of a gene, designated *hmt1*, that complements a Cd-sensitive *S.pombe* mutant deficient in production of the HMW PC−Cd−S^{-2} complex. The amino acid sequence deduced from an *hmt1* cDNA exhibits similarity with ABC (ATP-binding cassette)-type membrane transport proteins. Members of this family include the polypeptides encoded by the mammalian *mdr* (multiple drug resistance) and *cftr* (cystic fibrosis transmembrane regulator) genes, the *S.cerevisiae STE6* (sterile 6) locus, and a number of bacterial genes (reviewed by Juranka *et al.*, 1989). Additionally, we present evidence indicating that the *hmt1* gene product is localized to the vacuolar membrane. These findings link vacuolar transport with formation or stabilization of the HMW PC−Cd−S^{-2} complex.

Results

A Cd-sensitive mutant

As a first step in identifying the genes involved in PC-mediated heavy metal tolerance, Cd-sensitive mutants were isolated from an ethylmethanesulfonate mutagenized Sp223 (*ade6*$^-$, *leu1*$^-$, *ura4*$^-$) progenitor strain. A number of these mutants showed decreased viability at Cd concentrations 30-fold lower than those tolerated by Sp223 (0.025 mM versus 0.75 mM $CdSO_4$). PCs in cell extracts derived from Cd-induced cell cultures were resolved by gel filtration chromatography. Figure 1 diagrams typical elution profiles produced by Sp223 and LK100, one of the Cd-sensitive mutants. Two peaks containing bound Cd that represent the LMW and HMW PC−Cd complexes are resolved from the Sp223 extract. The LK100 extract yields an equivalent amount of the LMW complex but a much reduced level of the HMW peak. The Cd-sensitive phenotype of this strain is probably due to the diminished synthesis of the HMW PC−Cd−S^{-2} complex.

Cloning of the hmt1 gene

An *S.pombe* genomic DNA library generated in the yeast plasmid vector pART1 was transformed into LK100 and transformants were selected on Cd-containing medium. Only a single plasmid, pGS3, rescued from a Cd-tolerant transformant, was found to restore Cd tolerance and accumulation of the HMW PC−Cd−S^{-2} complex upon re-transformation into LK100 (Figure 1). The Sp223 extract contains a significant proportion of the metal in the LMW complex. In contrast, most of the bound Cd in an extract derived from LK100 harboring pGS3 is found in the HMW fractions which are also rich in acid labile sulfide (data not shown). Furthermore, both Sp223 and LK100 bearing pGS3 are more tolerant to Cd than Sp223 harboring pART1.

A 9 kb genomic DNA insert was found within pGS3. To delimit the region complementing LK100, a number of deletion derivatives of pGS3 were generated. Complementation analysis of LK100 transformants bearing these deletion constructs limited the region capable of restoring Cd tolerance to a 3.0 kb insert. On Northern blots of *S.pombe* RNA, this 3.0 kb DNA fragment hybridizes to a band corresponding to 2700−2800 nucleotides in length (Figure 2). Exposure to Cd, however, does not increase the

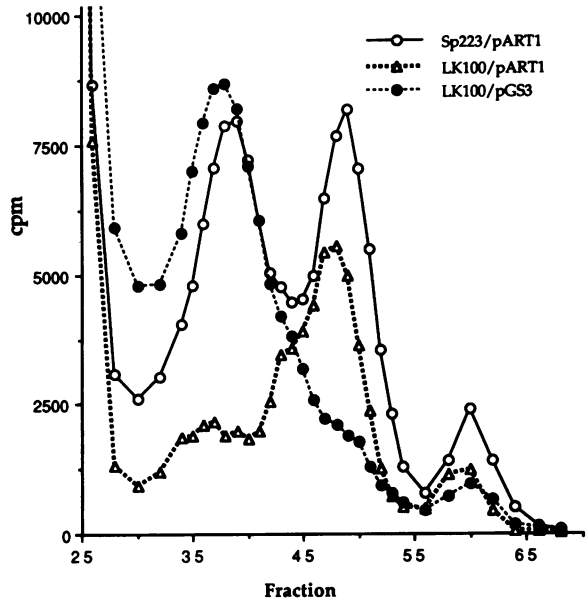

Fig. 1. PC−Cd complexes present in Sp223 and LK100 strains. Sephadex G-50 gel filtration profiles of the LMW and HMW PC−Cd complexes present in extracts derived from cultures exposed to 0.2 mM $CdSO_4$. Detection of the PC−Cd complexes by atomic absorption spectroscopy, or through inclusion in the sample of a ^{109}Cd tracer, does not significantly alter the shape of the elution profile (not shown). Samples were derived from Sp223 and LK100 strains harboring pART1, and LK100 containing pGS3. HMW and LMW PC−Cd complexes are centered on fractions 38 and 48, respectively. The peak preceding the PCs corresponds to Cd non-specifically bound to macromolecules in the extract. Spheroplast extracts from which cell wall components and nuclei have been removed exhibit a drastic reduction in the amount of Cd eluting in the void volume (not shown). Unbound Cd elutes in fractions 56−65.

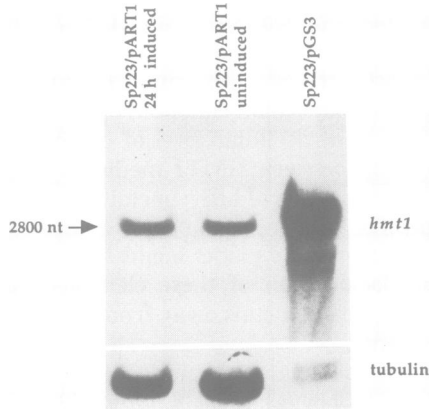

Fig. 2. *hmt1* transcript accumulation is not inducible by Cd. Autoradiograms of a Northern blot generated using RNA derived from cells grown in the presence or absence of 0.2 mM $CdSO_4$. Each of the two Sp223/pART1 lanes (Cd-induced and non-Cd-induced) contains 10 μg of poly(A)$^+$ RNA whereas only 3 μg were loaded in the Sp223/pGS3 lane. The radioactively labeled 3 kb insert of pDH22 was used as the hybridization probe in the top panel. After stripping the blot of this probe, it was rehybridized with a radioactively labeled α-tubulin gene fragment used as a control, producing the autoradiogram shown in the bottom panel.

level of this RNA species. In strains harboring pGS3, this transcript is found in great excess. Hence, the enhanced production of the HMW PC−Cd−S^{-2} complex, and the increased resistance to Cd, probably result from an overproduction of a pGS3-encoded product. Identified as involved in *h*eavy *m*etal *t*olerance, we named this gene *hmt1*. Southern analysis of DNA isolated from purified chromosome bands shows that this gene resides on chromosome III (Figure 3).

A cDNA library, generated using RNA isolated from Sp223, was probed for the presence of *hmt1* hybridizing sequences. One phage isolate was found to carry a 2.75 kb insert, which is approximately the size expected of a full length cDNA. This cDNA was inserted downstream of the *S.pombe adh1* promoter in pART1 to produce pDH35. LK100 transformed with pDH35 acquires a Cd tolerant phenotype and accumulates the HMW PC−Cd−S^{-2} complex to the same degree as transformants bearing pGS3 (data not shown). This indicates that pDH35 contains a functional *hmt1* cDNA clone.

hmt1 sequence

The sequence of the cDNA contains a single long open reading frame (ORF) in one strand and no ORF longer than 200 bp in the other (Figure 4). The sequence flanking the ATG codon preceding this long ORF matches the consensus eukaryotic translation initiation motif (Kozak, 1986). Provided this ATG encodes the amino terminal methionine, a protein 830 amino acids (aa) in length (mol. wt 90.5 kDa) would be produced upon translation.

To ascertain that restoration of Cd tolerance to LK100 by overexpression of a plasmid-borne *hmt1* gene was not due to extragenic suppression of Cd-sensitivity, disruption of the wild type allele in Sp223 via homologous recombination was attempted several times. Linear DNA constructs, in which all or most of the *hmt1* coding region had been substituted with a 1.6 kb fragment containing the *S.cerevisiae URA3* gene, were transformed into Sp223. More than 200 uracil prototrophic clones were examined by Southern hybridization. In about a quarter of those examined, the gene disruption construct had integrated into the *hmt1* locus. However, in all cases, a non-disrupted copy of the *hmt1* gene was also present, suggesting that duplication of the region

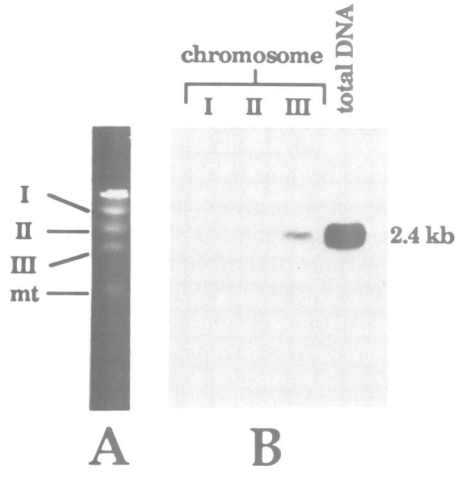

Fig. 3. The *hmt1* locus resides on chromosome III of *S.pombe*. The DNA from lysed *S.pombe* spheroplasts was separated by pulsed field gradient gel electrophoresis. **Panel A** is a photograph of an ethidium bromide stained CHEF gel. *S.pombe* chromosomes are labeled as I, II and III and mitochondrial DNA as mt. DNA extracted from agarose plugs containing the chromosomal bands was digested with *Bam*HI and *Pst*I endonucleases and subjected to Southern blotting. The autoradiogram in **panel B** is generated when this filter is hybridized to an *hmt1* probe. Single bands are seen in the lanes containing total DNA and chromosome III DNA. When rehybridized to *ade2* DNA, a chromosome I specific probe, an autoradiogram of this same blot displayed a signal only in the chromosome I and total DNA lanes (not shown), indicating that the signal detected in the chromosome III lane with the *hmt1* probe is not due to contamination with total genomic DNA. This indicates that *hmt1* is on chromosome III.

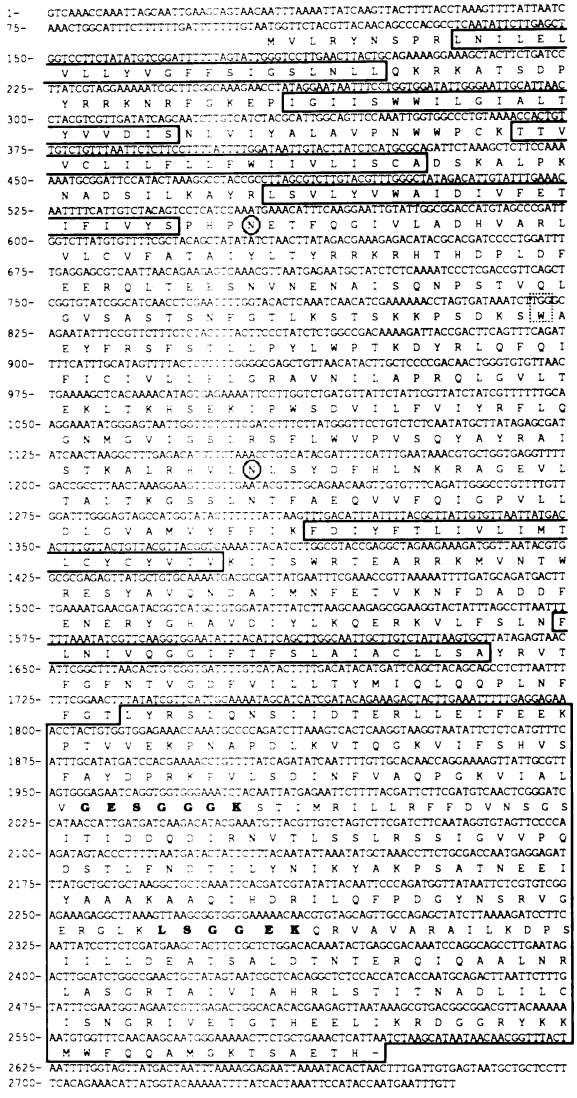

Fig. 4. *hmt1* nucleotide sequence and deduced protein sequence. DNA sequence of the cDNA insert of pDH30 reveals a long ORF, starting with the ATG at bp 107, which could encode a 90 500 Da protein. Analysis of the aa sequence indicates the presence of 6−10 transmembrane domains (narrow boxes containing only aa sequence) in the amino terminal region of the protein. In keeping with structures proposed for other ABC membrane proteins, only six of the membrane spanning domains are indicated. The two Asp residues residing in sites matching the consensus motif susceptible to N-linked glycosylation (encircled) might be modified. The large box encompassing the carboxyl terminal region contains the bipartite NBS (bold characters). The Trp codon that was found mutated to a translation termination TAG triplet in the LK100 *hmt1*$^-$ allele is indicated by a dashed box. The nucleotide sequence of *hmt1* has been deposited in the EMBL database under the accession number Z14055.

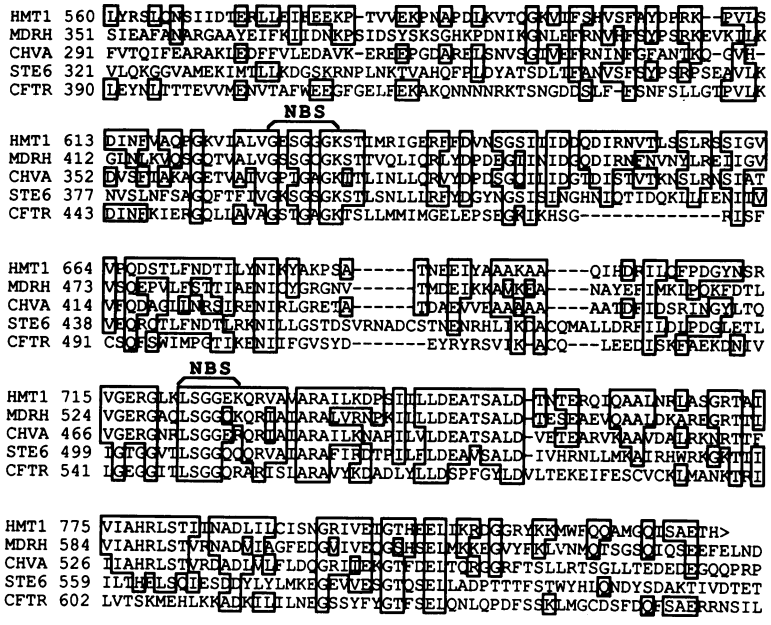

Fig. 5. Alignment of the HMT1 amino acid sequence flanking the NBS with corresponding regions of other ABC-type transporters. The sequences flanking the NBS of HMT1 (the carboxyl terminal region boxed in Figure 4) are aligned with corresponding regions of four ABC-type membrane proteins. The boxed aa represent identity of the HMT1 sequence to proteins encoded by human *mdr3* and *cftr*, *Agrobacterium tumefaciens chvA* and *S.cerevisiae STE6* genes. Dashes indicate gaps introduced in the sequence to maximize alignment. In all cases identity is limited to the sequences flanking the NBS: no significant similarity can be detected with the hydrophobic domain. The three eukaryotic proteins contain a second NBS domain which also exhibits homology with HMT1 (not shown); in all cases it is the most amino terminal of these two domains that exhibits the highest level of aa identity.

containing this gene had taken place (data not shown). In lieu of a bona fide gene disruption, the fragment bearing the *hmt1*$^-$ mutant allele was cloned from LK100. Indeed, this DNA fragment was unable to restore Cd tolerance to LK100. Sequence analysis indicated that a single G/C to A/T transition had altered the TTG triplet coding for Trp253 to a TAG termination codon (Figure 4). Thus, the molecular basis for the Cd-sensitive phenotype of LK100 is probably a severely reduced synthesis of the full length polypeptide, HMT1, encoded by the *hmt1* ORF.

HMT1 protein

Computer aided analysis of the aa sequence deduced from the cDNA suggests that *hmt1* encodes a protein with two well defined domains. The amino terminal region of the polypeptide (aa 1−500) is quite hydrophobic and contains multiple putative membrane spanning regions. The algorithm of Eisenberg *et al.* (1984) infers six membrane spanning domains, whereas the method of Klein *et al.* (1985) indicates the presence of 10 transmembrane helices. In the absence of structural data, the exact number of membrane spanning segments remains unknown. The sequence of the region containing the amino terminal transmembrane helix matches the consensus sequence for the eukaryotic signal peptide, including a possible cleavage site fulfilling von Heijne's −1,−3 rule at aa 27 (von Heijne, 1985). These features suggest that the *hmt1* gene product is an integral membrane protein.

The carboxyl terminal region is more hydrophilic and contains a putative composite nucleotide binding site (NBS) (Walker *et al.*, 1982). A search of protein sequence data banks with the HMT1 aa sequence indicated that a carboxyl terminal segment containing the NBS displays sequence similarity with polypeptides belonging to a large family of membrane transport proteins (Figure 5). In all cases, aa sequence identity of these proteins with HMT1 is confined to a 200−250 aa region surrounding the NBS. The P-glycoproteins encoded by the human *mdr3* and mouse *mdr2* genes exhibit the highest level of sequence similarity with HMT1. The *Agrobacterium tumefaciens chvA* gene product is next, followed by the proteins encoded by other mammalian *mdr* genes. Other eukaryotic proteins exhibiting similarity to HMT1 include the budding yeast STE6 polypeptide, the MDR-like protein of *Plasmodium falciparum*, and the human CFTR. Although all of the eukaryotic polypeptides also contain polytopic membrane spanning regions upstream of the NBS domains, these regions exhibit little or no sequence similarity amongst themselves or with the HMT1 hydrophobic domain. Figure 5 illustrates the level of aa identity among HMT1 and selected members of the ABC-type membrane transporter family. The sequences presented in Figure 5 by no means cover all of the genes belonging to this group, which now number over 40 if bacterial proteins containing an NBS but without a membrane domain are included.

Subcellular fractionation of S.pombe

Analysis of cells grown in the presence of cadmium provided a clue to the intracellular location of HMT1. *S.pombe* strains harboring an *hmt1*-expressing multicopy plasmid (pGS3 or pDH35) exhibit more resistance to Cd, and depending on growth conditions, accumulate 24−147% more ^{109}Cd tracer per cell than do the same strains bearing the vector alone (data not shown). This HMT1 associated increase in heavy metal accumulation suggests that this protein does not function as a plasma membrane Cd efflux pump, as its overexpression would probably have the opposite effect of reducing intracellular Cd levels. On the other hand,

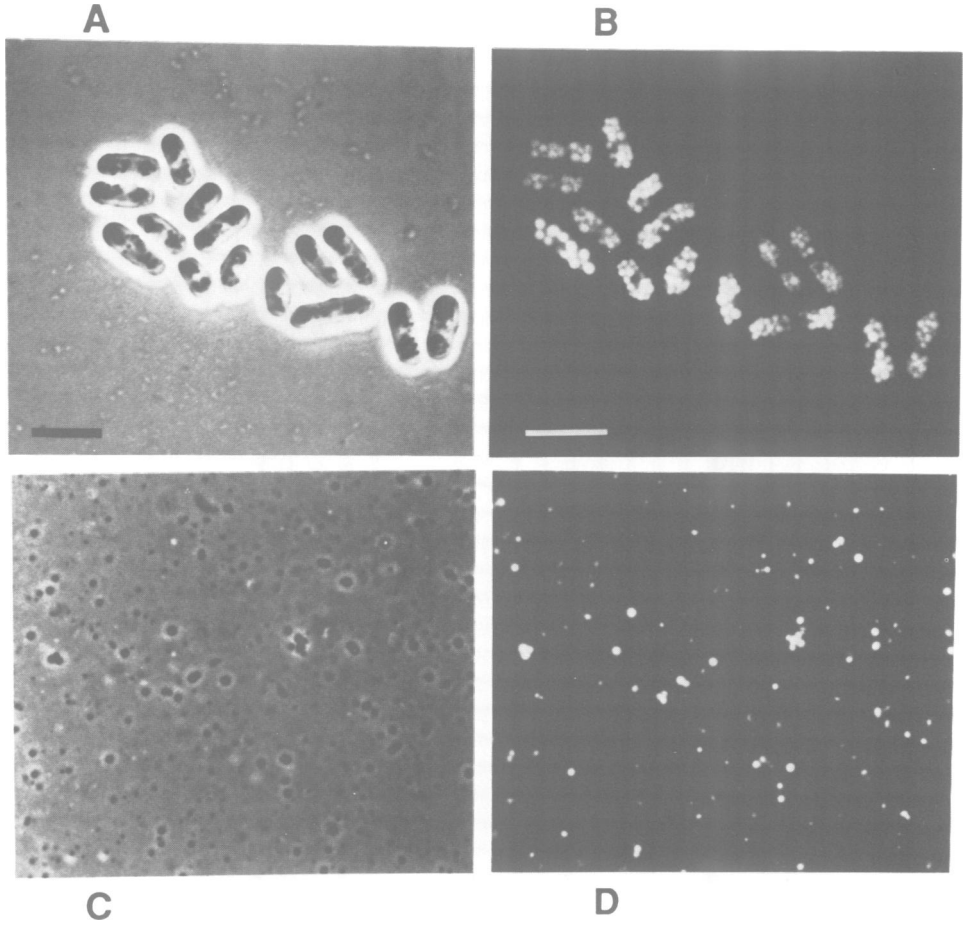

Fig. 6. *ade6*$^-$ *S.pombe* strains (deficient in 5-aminoimidazole ribonucleotide carboxylase) accumulate a red fluorescent pigment in the vacuole. As seen in panel **A**, phase contrast microscopy of *S.pombe* cells immobilized in 25% gelatin reveals few intracellular features. Epifluorescence microscopy of the same cells (an Sp223 *ade6*$^-$ culture grown to late log phase in SD supplemented with 2 μg/ml adenine), identifies fluorescent vesicles (panel **B**) matching the descriptions of vacuoles derived from electron and light microscopy of fixed cells (Robinow *et al.*, 1989). Retention of fluorescence by these vesicles after spheroplast lysis greatly facilitated tracking of vacuoles during subcellular fractionation. The end result of the fractionation procedure (detailed in Materials and methods) is a pellet composed mostly of these fluorescent vesicles (panels **C** and **D**). Bar is 5 μm.

HMT1-aided sequestration of Cd into a membrane bound compartment, such as the vacuole, would be consistent with the observed increase in cell associated Cd.

One approach for testing this hypothesis would be to purify *S.pombe* vacuoles and determine if the HMT1 protein is sorted to this organelle. Although a method describing the isolation of fission yeast vacuoles was unavailable in the literature, and procedures used in the isolation of *S.cerevisiae* vacuoles were not successful in our hands, promising results were obtained after modification of a purification protocol decribed for *N.crassa* vacuoles (Bowman and Bowman, 1988). Tracking the vacuoles during this procedure was aided enormously by the fact that yeast strains bearing mutations in certain genes of the purine biosynthetic pathway accumulate a fluorescent red pigment in the vacuole when cultured in media low in adenine (Weisman *et al.*, 1987). Cultures of the *ade6*$^-$ Sp223 progenitor strain, as well as the LK100 mutant, develop a reddish hue when grown to late log phase in minimal medium containing 2 μg/ml adenine. As shown in Figure 6B, epifluorescence microscopy reveals the presence of numerous small fluorescent vesicles in these cells. These vesicles are similar in size and number to the polyphosphate-rich vacuoles observed in fixed *S.pombe* cells by light and electron microscopy (reviewed by Robinow and Hyams, 1989). Vacuolar morphology in the mutant, or the wild type, is not affected by growth on Cd. Mechanical lysis of spheroplasts in buffers of high osmolarity releases the fluorescent vesicles, which can then be purified. Under microscopic examination, the final fraction obtained from this procedure indicates that it is composed mostly of fluorescent vesicles indistinguishable from those found in intact cells (Figure 6D). Table I shows that this fraction is indeed highly enriched for vacuolar enzyme activities such as α-mannosidase and carboxypeptidase Y, but impoverished in glucose-6-phosphate dehydrogenase activity, indicating low levels of contamination with cytoplasmic proteins. These characteristics suggest that the fractionation procedure resulted in a significant enrichment of intact vacuoles.

Intracellular localization of HMT1

Purification of the HMT1 protein from *Escherichia coli* expressing the *hmt1* gene was attempted with the intention of raising antibodies. However, as is often the case with polytopic membrane proteins, overproduction of HMT1 in the bacterial system has been problematic. An alternative

Table I.

	Enzyme activity (μmol/min/mg protein)		
	α-Mannosidase	CPY	G-6-P DH
Vacuoles	26.3	17.3	3.5
Total	0.73	0.06	188
Vacuoles/total	36	288	0.02

The fraction containing purified fluorescent vesicles is enriched in vacuolar marker enzymes. The activities of marker enzymes were measured in aliquots of the pellet obtained from the vacuolar purification procedure (vacuoles) and of the cellular extracts cleared by centrifugation at 4000 *g* (total). The α-mannosidase activity is associated with vacuoles of a number of fungi. Carboxypeptidase Y (CPY), as shown in *S.cerevisiae*, is sorted to the vacuoles, and glucose 6-phosphate dehydrogenase (G-6-P DH) is a cytoplasmic enzyme. A cytoplasmic inhibitor of CPY, of the type described in *S.cerevisiae* (Jones, 1991), could result in underestimation of this activity in the total extract. This may account for the apparent greater purification of CPY relative to α-mannosidase.

approach, used successfully with the human P-glycoprotein (Germann *et al.*, 1989), involves tagging the polypeptide of interest with a protein for which antibodies are readily available. For this purpose, the *hmt1* coding region was fused in frame to the 5′ terminus of the *E.coli lacZ* ORF and subcloned into the pART1 yeast expression vector to produce pDH40. LK100 transformed with pDH40 was able to grow on medium containing 0.2 mM Cd, although more slowly than Sp223 harboring pART1. This indicates that the fusion protein between HMT1 and β-galactosidase (βGAL) is still functional. Hence, a reasonable assumption is that the fusion protein is also sorted correctly.

In a preliminary test to address the question of localization of the HMT1−βGAL chimeric protein, cleared extracts prepared from yeast spheroplasts were separated into soluble (S100) and particulate (P100) fractions by ultracentrifugation at 100 000 *g*. Immunoblot analysis of these fractions using monoclonal antibodies directed against the βGAL moiety produced a signal that is present exclusively in extracts derived from pDH40 transformants, and then only in the P100 fraction, with no detectable binding of the anti-βGAL antibodies to proteins in the soluble supernatant (data not shown). This is consistent with the prediction that HMT1 is a membrane associated protein.

To determine if HMT1 resides in the vacuolar membrane, yeast cells producing the HMT1−βGAL chimeric protein were fractionated as described in Materials and methods. The proteins in various fractions were separated by SDS−PAGE and analyzed by immunoblotting. As seen in Figure 7, most of the chimeric protein is found in membranes purified from the vacuole fraction, suggesting that HMT1 is sorted to the vacuolar membrane. The weak signal that can be detected in the vacuole-depleted P100 membrane fraction probably originates from vacuoles not pelleted by the preliminary 16 000 *g* centrifugation (some fluorescent vesicles are discernible in the supernatant at this point), or from vacuolar membranes derived from lysed organelles. Alternatively, it could represent HMT1−βGAL protein present in other cellular membranes while in transit to the vacuolar membrane.

The mol. wt (185−200 kDa) of the proteins detected by antibodies directed against βGAL is approximately that predicted for the chimeric fusion protein (205 kDa). The

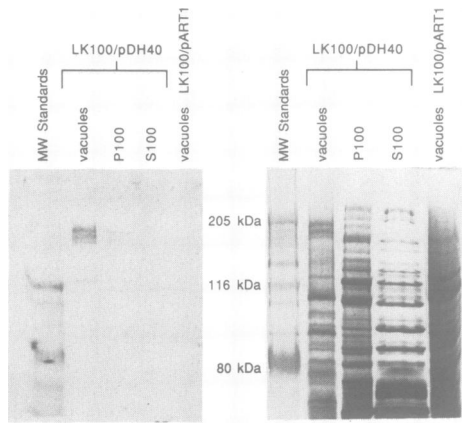

Fig. 7. The HMT1−βGAL fusion protein is localized to the vacuolar membrane. Immunoblot of a 7.5% SDS−PAGE gel containing proteins from various subcellular fractions derived from LK100 cells bearing either pDH40 or pART1. The bands were developed with a monoclonal antibody directed against the βGAL moiety and visualized with a second anti-mouse IgG antibody linked to alkaline phosphatase. Vacuolar membranes were obtained by lysis of purified vacuoles and centrifugation at 100 000 *g*. The S100 and P100 fractions represent the supernatant and pellet, respectively, derived by separation at 100 000 *g* of the vacuole-depleted 16 000 *g* supernatant (see Materials and methods). The right panel is a silver stained replica of the gel used for the immunoblot on the left. The mol. wt markers are high range pre-stained SDS standards from Bio-Rad; the strong signals in this lane of the immunoblot represents binding of antibodies to the *E.coli* βGAL 116 kDa MW standard and its degradation products.

significance of the multiple bands seen on the immunoblot is unclear at this point. A number of possibilities arise. Certain membrane proteins, including the ABC-type transporters, are not readily solubilized by SDS under harsh conditions (>75°C); rather, they form aggregates that run as high mol. wt complexes. This appears to be the case with the HMT1−βGAL fusion protein, which must be incubated in loading buffer at 55°C. If these relatively mild denaturing conditions do not completely unfold the chimeric protein, the resulting isoforms could then be detected as multiple bands. Supporting this conjecture is the finding that in boiled extracts, the faint signal detected below the high MW aggregate is in two bands corresponding to 200 kDa and 205 kDa (data not shown). Alternatively, post-translational modifications, such as glycosylation or proteolytic cleavage, may be responsible for this pattern. As indicated previously, the exact topological orientation of different regions of HMT1 is not known; however, two possible sites for N-linked glycosylation are found in the protein sequence (see Figure 4). Lastly, the multiple bands may represent products of protein degradation incurred during preparation.

Discussion

The HMW PC−Cd−S^{-2} complex is essential for a wild type level of Cd tolerance in the fission yeast. Although there are no firm data to conclude that this is the case with plants, *L.esculentum* (Reese *et al.*, 1992) and *Brassica juncea* (Speiser *et al.*, 1992) have been found to accumulate the HMW PC−Cd−S^{-2} complex when exposed to Cd. In addition, Cd tolerance in *Silene vulgaris* ecological isolates correlates with an increase of sulfide associated with the PC−Cd complex (Verkleij *et al.*, 1990). Together, these

findings suggest that the sulfide-containing complex may also contribute to enhanced tolerance in higher plants. It has been shown that the HMW complex can be derived *in vitro* from the LMW form by addition of sulfide (Reese and Winge, 1988). In this work, we have identified the *hmt1* gene as an *in vivo* requirement for the accumulation of the HMW $PC-Cd-S^{-2}$ complex.

As with most stress responses, synthesis of PCs is induced in response to a specific environmental challenge. This apparently is controlled via allosteric activation of PC synthase by heavy metal ions, and there is no indication that transcriptional modulation is involved (Löffler *et al.*, 1989). Likewise, we have found that the *hmt1* transcript level does not increase upon Cd induction of PC accumulation. It is possible that regulation of PC-mediated heavy metal tolerance occurs largely by post-transcriptional and/or post-translational mechanisms, in contrast to the metallothionein genes which are transcriptionally activated by heavy metal ions (Hamer, 1986).

HMT1 as a vacuolar membrane transport protein

The aa sequence deduced from a functional *hmt1* cDNA suggests that the translation product is a polytopic membrane protein with structural and aa sequence similarities to eukaryotic and prokaryotic ABC-type membrane transport proteins. This family of transporters mobilizes proteins and other compounds across membrane barriers, representing a highly versatile alternative to the vesicular transport pathway. Eukaryotic cells exploit this resource in various ways, such as the cellular export of ions (CFTR) (Riordan *et al.*, 1990), and pheromones (STE6) (McGrath and Varshavsky, 1989; Kuchler *et al.*, 1989), the transport of peptide fragments into the endoplasmic reticulum (RING4) (Trowsdale *et al.*, 1990), or of proteins into peroxisomes (PMP70) (Kamijo *et al.*, 1990). The ABC-type transporters also function effectively in mediating cellular resistance to a number of toxins, exemplified by multiple drug resistance in mammalian cells, arsenic tolerance in bacteria (Chen *et al.*, 1986) and chloroquine resistance in *Plasmodium falciparum* (Cowman *et al.*, 1991).

Analysis of subcellular fractions derived from fission yeast bearing an *hmt1'–lacZ* translational fusion shows that the HMT1–βGAL chimeric protein is sorted to the vacuolar membrane. This DNA construct also confers Cd tolerance to LK100, indicating that the fusion protein remains at least partially functional and supporting the contention that it is sorted correctly. Additionally, cells overexpressing the wild type *hmt1* allele accumulate more, rather than less, Cd ions when exposed to the metal, suggesting that HMT1 plays a role in intracellular sequestration of Cd rather than cellular efflux of the metal. All three observations are consistent with a vacuolar membrane location for HMT1.

The sequences directing the HMT1 polypeptide to the vacuoles are not obvious. The signal sequence of *S.cerevisiae* repressible alkaline phosphatase (ALP) can direct chimeric polypeptides to the budding yeast vacuolar membrane (Klionsky and Emr, 1990). The putative signal peptide in the amino terminus of HMT1 exhibits no homology with the ALP counterpart. However, a Thr-Val-Val-Cys motif in the fourth membrane spanning domain of HMT1 is also present in the ALP signal peptide. It remains to be tested if this limited aa identity is significant.

Most eukaryotic ABC-type proteins identified are sorted to the plasma membrane, and display a tandemly duplicated structure. Sequence analysis suggests that the genes encoding these peptides arose by duplication and fusion of an ancestral gene encoding a single NBS domain preceded by numerous membrane spanning regions. Recently, eukaryotic ABC-type transporters displaying the ancestral monomeric structure have been reported, and in each case there is evidence indicating that these proteins are localized to intracellular membranes. PMP70 has been localized to the peroxisomal membrane (Kamijo *et al.*, 1990), RING4 may reside in the endoplasmic reticulum (Trowsdale *et al.*, 1990) and HMT1 is probably sorted to the vacuolar membrane. Although this represents a small sample, it suggests a correlation between protein structure and cellular location. On the other hand, the *P.falciparum pfmdr1* gene product, which has a duplicated structure, is sorted to the vacuolar membrane (Cowman *et al.*, 1991).

HMT1 function and heavy metal tolerance

Subcellular compartmentalization of metabolic processes and compounds is the hallmark of eukaryotic cells. It is well established that a wide variety of hydrolases are sequestered within the fungal vacuole. Additionally, cytoplasmic homeostasis of a number of amino acids and inorganic ions, such as Ca^{2+}, Zn^{2+} and polyphosphate, is mediated in part by storage of these compounds in the vacuole. By analogy, it is conceivable that the *S.pombe* vacuoles play a central role in PC-mediated heavy metal detoxification. Supporting this contention is the finding that most of the Cd ions taken up by tobacco cells accumulate in the vacuole, as do the PCs synthesized in response to this stress (Vogeli-Lange and Wagner, 1990).

Specific transporters residing in the fungal vacuolar membranes are responsible for the maintenance of vacuolar pools of arginine and Ca^{2+} (see Klionsky *et al.*, 1990 for a review of *Saccharomyces* sp. and Davis, 1986 for *N.crassa*). The finding that a putative transport protein, required for HMW $PC-Cd-S^{-2}$ complex accumulation, is localized to the vacuolar membrane in *S.pombe* adds a key element to the working model of PC-mediated heavy metal tolerance. It is known that cellular uptake of Cd induces PC synthesis via allosteric activation of PC synthase. The PCs thus produced would chelate the free metal ions, probably by forming the LMW complex. We hypothesize that this species is then transported across the vacuolar membrane, where sulfide is incorporated in the lumen of the vacuole to generate the HMW complex. In this model the LMW PC–Cd complex would function as a scavenger and carrier of cytoplasmic Cd, whereas the HMW complex would deal with storage. This role is consistent with the increased stability and metal-binding capacity of the HMW complex.

The fact that strains overexpressing *hmt1* are more tolerant to Cd, and produce more of the HMW complex than the wild type strain, suggests that HMT1-mediated transport may be a rate limiting step in the formation of the HMW $PC-Cd-S^{-2}$ complex. What is not known, however, is the exact substrate mobilized by this protein. The most likely candidates are the LMW and HMW PC–metal complexes. If conversion of the LMW complex to the HMW form by sulfide incorporation takes place in the vacuole, a block in PC transport across the vacuolar membrane would explain the lack of HMW complex accumulation observed in LK100.

A second possibility is that the HMW complex is synthesized in the cytoplasm, but is unstable in this environment and requires sequestration in a membrane bound compartment. Alternatively, PC translocation across the vacuolar membrane might not depend on HMT1, but rather it is sulfide, a sulfide precursor, or an enzyme involved in sulfide incorporation into the LMW PC−Cd complex, which is the substrate of HMT1.

Finally, it is tempting to speculate that HMT1 function may also be related to GSH-mediated transport. A large number of toxic compounds are tagged for breakdown, or export from the cell, by modification via glutathione-S-transferases (reviewed by Pickett and Lu, 1989). West (1990) has proposed that recognition of the GSH tag on these adducts by one, or a few, membrane transport proteins, would eliminate the need for a specific transporter for each GSH conjugate shunted across a membrane. Since PC peptides contain a carboxyl terminal GSH residue, the intriguing possibility of a similar mode of transport used by PCs and GSH is conceivable.

Expansion in industrial and mining activities during recent decades, as well as changes in agricultural practices, have resulted in a significant increase in heavy metal contamination of groundwater and soils. In their assessment of global pollution, Nriagu and Pacyna (1988) concluded that the annual toxicity of metal emissions exceeds that of radioactive and organic wastes combined. Soil and water contamination leads to uptake of Cd and Pb by crop plants, and ultimately, human and animal consumption. Elucidating the biochemical and cellular processes mediating heavy metal tolerance in plants should aid in the design of genetically modified crops with diminished uptake of these toxins, or which restrict their accumulation to non-consumable tissues. The phenotype conferred by overexpression of *hmt1* in *S.pombe* suggests that the overproduction of a functional HMT1 protein, or a plant homolog, in non-consumable tissues of crop plants might be a viable strategy for reducing the dietary uptake of heavy metals.

Materials and methods

Isolation and characterization of S.pombe mutants

Yeast cultures were grown at 30°C. The minimal and rich media used were, respectively, SD [6.7 g yeast nitrogen base (Difco), 20 g glucose/l] and YG [5 g yeast extract (Difco), 20 g glucose/l]. Where appropriate, nutritional supplements were added at 20 μg/ml for nucleotide bases and 100 μg/ml for amino acids unless otherwise indicated.

Sp223 (*h*$^-$, *ade6*-216, *leu1*-32, *ura4*-294) cultures were treated with ethylmethanesulfonate as described by Sherman *et al.* (1986) and Cd-sensitive mutants were screened by individual patching onto YG agar plates (1.5% Gibco Select agar) containing 0, 0.1, 0.2 and 0.4 mM $CdCl_2$. Isolates that were Cd-hypersensitive were checked to ensure the absence of additional auxotrophic requirements. PC accumulation in these mutants was analyzed by gel filtration chromatography. *S.pombe* cells were grown to an OD_{595} of 0.4 in YG (or SD for strains with plasmids) and induced by addition of $CdCl_2$ or $CdSO_4$ to a final concentration of 0.2 mM. After a 30 h induction period, the cells were harvested by centrifugation at 1000 *g* for 5 min, washed twice with 50 mM Tris pH 7.8, and resuspended in 1 vol of the same buffer. Cells were vortexed with glass beads until 80−90% of the cells had lysed (as determined by phase contrast microscopy). Extracts were cleared by centrifugation for 5 min at 15 000 *g*. An aliquot corresponding to 2 mg protein (measured by the Bio-Rad laboratories dye binding assay) was labeled by addition of 0.25 μCi ^{109}Cd and separated on a Sephadex G-50 column equilibrated and run with 50 mM Tris, pH 7.8. Cd-containing peaks were detected by counting column fractions by liquid scintillation.

Library construction and isolation of complementing clones

DNA was isolated from freeze−thawed spheroplasts and resuspended in 50 mM Tris pH 8.0, 100 mM NaCl and 1 mM EDTA. SDS and proteinase K were added to 1% and 20 μg/ml respectively. The samples were incubated at 55°C for several hours, phenol extracted, ethanol precipitated and treated with RNase A. Chromosomal DNA from Sp223 was partially digested with *Sau*3A and size-fractionated by sucrose density gradient centrifugation. DNA fragments of ~10 kb were collected and ligated to the *S.pombe* expression vector pART1 (Russell, 1989) that had been cleaved with *Bam*HI and dephosphorylated. A library of ~10^5 clones was obtained and used for DNA transformation as described for *S.pombe* (Beach and Nurse, 1981).

Chromosome assignment of HMT1

The three *S.pombe* chromosomes were separated by pulsed field gel electrophoresis following the procedure of Smith *et al.* (1987). The chromosomal bands were cut out from the gel and the DNA extracted using Geneclean (Bio 101) according to instructions supplied by the manufacturer. The purified genomic DNA was cleaved with *Bam*HI and *Pst*I and subjected to Southern analysis with pGS3 as a hybridization probe.

Plasmid constructs

The 9.0 kb insert within pGS3 was subjected to restriction endonuclease digestion to generate various smaller fragments which were religated back into the vector pART1. From complementation analysis of this set of subclones, the complementing region was delimited to a 5.5 kb *Sal*I subclone (pDS21). Plasmid pDS21 was cut immediately upstream or downstream of the genomic insert and treated with Bal31 exonuclease. These truncated fragments were isolated and reinserted in their original orientation into pART1. Complementation analysis further delimited the size of the complementing insert to 3.0 kb (pDH22). This fragment was used as a probe for Northern analysis and cDNA clone isolation.

Two gene disruption constructs were made. A 1.5 kb *Bam*HI−*Bgl*II, or a 5 kb *Bam*HI−*Bgl*II fragment containing the *hmt1* coding region, was deleted from pUC19 derivatives bearing the pGS3 insert or a subclone containing a *Sal*I fragment. The deleted regions were replaced by a 1.6 kb DNA fragment containing the *S.cerevisiae URA3* gene. These constructs were linearized by cleavage with an enzyme cutting in the vector prior to transformation into Sp223. DNA isolated from uracil prototrophic colonies was analyzed by Southern blot analysis.

Plasmid pDH35, which complements LK100, was generated by ligating a *Sac*I−*Hin*cII fragment containing the *hmt1* cDNA insert in pDH30 (see cDNA cloning section) behind the *adh*1 promoter in pART1. To produce a fusion protein, the cDNA insert of pDH35 was cleaved at an *Ase*I site (AT*TAA*T), containing the translational stop codon of the *hmt1* ORF, and ligated to the 5′ terminus of an *E.coli lacZ* ORF lacking the translational start codon. The predicted translation product would be a chimeric HMT1−βGAL protein consisting of the entire HMT1 polypeptide fused to the amino terminus of βGAL.

Northern blots and cDNA bank

One liter cultures of *S.pombe* were induced with Cd for 24 h. RNA was extracted from the cellular pellet using a modification of the procedure described by Longemann *et al.* (1987). Briefly, cells were harvested by centrifugation at 4000 *g* and washed twice with ice-cold deionized water. The cellular pellet was resuspended in an equal volume of buffered 8 M guanidinium−HCl and vortexed with glass beads (0.45−0.50 mm in diameter) until 80−90% of the cells had lysed. One-third volume of chloroform was added and the glass beads and cell debris were pelleted by centrifugation at 10 000 *g*. The supernatant was then treated as described in the published procedure. Poly(A)$^+$ RNA was selected by oligo(dT) column chromatography and a Northern blot was generated. The probe used for detection of the *hmt1* transcript was the 3.0 kb genomic DNA insert of pDH22. A phage λgt10 cDNA library was prepared using a mixture of poly(A)$^+$ RNAs isolated from cultures grown in the presence of 0.2 mM $CdSO_4$ for 0, 15 and 24 h. The library was screened with the same probe used for Northern analysis (see above). Ten hybridizing plaques were identified and purified from a total of 30 000 screened. Phage DNA was prepared from six of these plaques, cleaved with *Eco*RI or *Not*I (restriction sites present in the adaptors used to ligate the cDNA into the λgt10 vector) and analyzed by Southern blotting. A 2.75 kb *Not*I fragment was isolated from the phage DNA exhibiting the largest insert and subcloned into the pBluescript SK and KS vectors (Stratagene) to generate plasmids pDH30 and pDH31 respectively.

Sequence determination and analysis

The sequence of the inserts in pDH30 and pDH31 was determined by the method of Sanger *et al.* (1977) using double stranded templates and 17 nucleotide primers complementary to the newly determined sequence. The *hmt1* DNA sequence was analyzed using the Intelligenetics program and the deduced protein sequence compared with the Swiss-prot data base. Hydrophilicity, possible membrane spanning domains and putative signature

sites of the HMT1 protein sequence were analyzed using the Intelligenetics program algorithms of Klein *et al.* (1985) and Eisenberg *et al.* (1984).

Cellular fractionation and protein analysis

Sp223 and mutant strains transformed with various plasmids were grown in SD medium in the presence or absence of Cd as described above. Cells were harvested and washed with water, and the pellet was resuspended in 10 ml 1.2 M sorbitol, 50 mM Na citrate, 50 mM $NaPO_4$, 40 mM EDTA, 10 mM mercaptoethanol (ME) and incubated for 10 min at RT. The cells were pelleted at 1000 *g* for 10 min and resuspended in 20 ml of the same buffer lacking EDTA and ME but containing 100 mg of Novozym (Novolabs). Digestion of the cell wall continued until >70% of the cells had spheroplasted, after which they were harvested at 1000 *g* and washed three times with WB (1.2 M sorbitol, 10 mM HEPES, pH 7.0). For purification of total membranes the spheroplast pellet was resuspended in lysis buffer (10 mM HEPES, 1 mM DTT, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA) and passed 10 times through a Dounce homogenizer. Cell debris and unbroken spheroplasts were pelleted at 4000 *g*, and the supernatant was subjected to 100 000 *g* in a Beckman SW41 rotor. The resulting membrane pellet was resuspended in lysis buffer containing 1% Triton X-100. Five milliliters of the P100 or S100 fractions were mixed with an equal volume of sample buffer, incubated at 55°C for 10 min and electrophoresed on a 7.5% polyacrylamide–SDS gel. The separated polypeptides were electroblotted onto nitrocellulose membranes and the chimeric fusion protein was detected by monoclonal antibodies directed against the βGAL moiety (Pharmacia).

Vacuoles were isolated using a modification of the procedure described by Bowman and Bowman (1988) for *N.crassa*. *S.pombe* spheroplasts were prepared as above. After the third WB wash, the cells were resuspended by vortexing in 10 ml of ice-cold 1.5 M sorbitol, 10 mM triethanolamine pH 6.9, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF. The partially lysed spheroplasts were transferred to a homogenizer and Dounced 10–15 times. The extract was cleared by centrifugation at 4000 *g* for 10 min and vacuoles were pelleted by centrifugation at 16 000 *g* for 30 min. The vacuolar pellet was gently resuspended with a homogenizer in 1 ml 1.5 M sorbitol, 10 mM HEPES pH 7.2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, loaded onto a 50–65% sucrose step gradient (50, 55, 60, 65% sucrose, 10 mM HEPES, 1 mM DTT) and centrifuged for 1 h at 20 000 r.p.m. in a Beckman SW28 rotor. The final vacuolar pellet residing under the 65% sucrose layer was resuspended in 0.5–1 ml of 2 M sorbitol, 10 mM HEPES, 1 mM DTT, aliquoted and frozen for subsequent analysis.

The protein concentration of vacuolar and supernatant fractions was determined using the Bio-Rad dye binding kit. Activities of α-mannosidase, carboxypeptidase Y and glucose-6-phosphate dehydrogenase were measured in aliquots of the resuspended vacuolar pellet and the 4000 *g* supernatant as detailed by Roberts *et al.* (1991). Because PMSF inhibits carboxypeptidase Y activity, this protease inhibitor was omitted from the lysis buffer in vacuolar preparations used to determine fractionation of carboxypeptidase Y activity.

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