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Dominant lethal mutations in the plasma membrane H$^+$-ATPase gene of *Saccharomyces cerevisiae*

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ABSTRACT **The plasma membrane H$^+$-ATPase of *Saccharomyces cerevisiae* is an essential protein that is required to establish cellular membrane potential and maintain a normal internal pH. An Asp-378 to Asn substitution at the residue phosphorylated during catalysis is dominant lethal when the *pma1-D378N* mutation is expressed along with a wild-type plasma membrane H$^+$-ATPase (*PMA1*) gene. Several mutations in the first two putative transmembrane domains are also dominant lethal. However, these dominant lethal mutants often appear to be innocuous, because they are frequently lost by gene conversion to the wild-type sequence during the process of introducing the mutant sequence and subsequently removing the wild-type gene. Loss of the mutation by gene conversion does not occur while introducing recessive lethal mutations. Cells carrying the wild-type *PMA1* gene on the chromosome and a dominant lethal mutation under the control of a *GAL1* promoter on a centromere-containing plasmid exhibit a galactose-dependent lethality. Indirect immunofluorescence staining using anti-Pma1 antibodies shows that induction of dominant lethal *PMA1* mutations leads to the accumulation of a number of intensely staining cytoplasmic structures that are not coincident with the nucleus and its immediately surrounding endoplasmic reticulum. These structures also accumulate the endoplasmic reticulum protein Kar2. Expression of the dominant lethal protein also prevents transport of the wild-type ATPase to the plasma membrane.**

The plasma membrane H$^+$-ATPase gene (*PMA1*) of the yeast *Saccharomyces cerevisiae* plays an essential role in the maintenance of intracellular pH and in the establishment of a large electrochemical gradient that is required for the transport of many nutrients into the cell [reviewed by Serrano (1)]. The study of the structure and function of this major plasma membrane protein is interesting not only in its own right but also because of its extensive structural similarity to the large family of cation ATPases in mammalian cells, including the Na$^+$,K$^+$-, Ca^{2+}-, and H$^+$,K$^+$-ATPases (1, 2).

One residue in *PMA1* that has been studied in detail is Asp378, which undergoes phosphorylation and dephosphorylation during catalysis (3, 4). The analogous residue in the mammalian Ca^{2+}-ATPase has been mutated and shown to be essential for catalytic activity (5). Surprisingly, Portillo and Serrano (6) reported that substitution of Asn for Asp-378 (D378N) in the yeast H$^+$-ATPase was nearly wild type in its behavior and formed a normally phosphorylated intermediate. Given the high degree of conservation of this Asp and its surrounding amino acids in all P-type ATPases, the difference in results between the yeast and mammalian ATPases was unexpected. It seemed possible to us that this surprising result could be explained by some secondary event that occurred after the D378N mutation was transformed into yeast.

We have investigated the fate of the *pma1-D378N* mutation after its introduction into yeast and have discovered that it is invariably lost by gene conversion with the wild-type allele during sequence shuffling if the mutant gene is constitutively expressed. We demonstrate, using a conditionally expressed *pma1-D378N* gene, that the mutation is dominant lethal and causes an arrest of cell growth with the accumulation of mutant and wild-type Pma1 proteins in novel cytoplasmic structures that also contain the endoplasmic reticulum protein Kar2. We show further that several other site-directed mutations are also dominant lethal and cause a similar cellular arrest.

MATERIALS AND METHODS

Media and Growth Conditions. The media and growth conditions for analyzing *pma1* mutants have been described (7, 8). Synthetic media containing dextrose (SD), lactic acid (SL), or galactose (SG) were used (9). Yeast cells were transformed by the lithium acetate method of Schiestl and Gietz (10).

Strain Construction. Strains SH122 and SH129, derived from Y55 (*HO gal3 MAL1 SUC1*), are heterozygous for a *LEU2* marked deletion of *PMA1* in which the 4.1-kb *Xho* I–*Bgl* II *PMA1* coding region was replaced with a 2.3-kb *LEU2* fragment (6) (see Fig. 1). Strains XZ611 and XZ634 are Gal$^+$ *ho leu2 ura3 trp1* segregants derived from segregants of a cross of strain Y55-296 (*HO leu2 ura3 gal3*) with strain JKM30 (*ho MATa leu2 ura3 trp1 GAL3 hmr::LEU2*). The diploid XZ635 was obtained by crossing strains XZ611 with XZ634.

Plasmids. Plasmid pSH30 was constructed by deleting a 2.1-kb *Asp*718 *PMA1* fragment from a pSH29 (7). *pma1-D378N* was made by *in vitro* mutagenesis as described (7, 11, 12) and subsequently subcloned as an *Asp*718 fragment into pSH30. All site-directed mutations of the first two transmembrane segments were made in pSN54 and subsequently subcloned into the *Eco*RV and *Bst*EII sites of pSN57 (11).

To place the *pma1* genes containing dominant lethal mutations under the control of galactose-inducible promoter, all mutations were subcloned into the centromere-containing plasmids pSN107 and pXZ03. An 11-kb *Aat* II–*Pvu* II fragment of pSH14 (7) and a 1.9-kb *Aat* II–*Pvu* II fragment of pRS61 (6) were ligated together to create pXZ03, containing the entire *PMA1* gene joined to a galactose-inducible promoter, in the *URA3* single-copy plasmid YCp50. pXZ09

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Abbreviations: HA, hemagglutinin; ER, endoplasmic reticulum; DAPI, 4′,6-diamidino-2-phenylindole.

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contains the D378N mutation. pSN107 is identical to pXZ03 except that the *URA3* gene on the original vector was deleted and replaced by inserting a *URA3* gene lacking its *Eco*RV site into the 3′ end of *GAL1*::*PMA1*. This modification made it easier to replace the *Eco*RV–*Bst*EII fragment with others containing site-directed mutations in the first two transmembrane domains.

Plasmid pXZ28 is identical to pXZ03 except that it carries a 33-nucleotide insert specifying the amino acids A, S, and the 9-amino acid hemagglutinin (HA) epitope (YPYDVPDYA). The epitope was introduced after the second amino acid of *PMA1* (X.Z. and J.E.H., unpublished data). This epitope insertion has no effect on the function of *PMA1* (X.Z. and J.E.H., unpublished data). Plasmid pXZ29 is identical to pXZ28 except that it carries the D378N mutation, inserted as described above for pXZ09. pXZ33 is identical to pXZ28 except the *URA3* gene was replaced with a *LEU2*-containing fragment.

DNA Analysis. DNA was extracted from yeast by standard methods (7, 11). Genomic DNA containing portions of *PMA1* was amplified by the polymerase chain reaction (PCR) (13) using Pyrostase DNA polymerase (Molecular Genetic Resources, Tampa, FL). The amplified DNA was sequenced with Sequenase (United States Biochemical) using the dimethyl sulfoxide method of Winship (14) or fmol-DNA sequencing system (Promega).

Cytological Analysis and Fluorescence Microscopy. Staining of fixed yeast cells by indirect immunofluorescence was carried out essentially as described by Davis and Fink (15). XZ635 was transformed with pXZ09 or with pSN107 derivatives containing different mutations in the first two transmembrane domains. The transformed strains were pregrown in 5 ml of SD-ura medium for 4 hr at 30°C. The cultures were then collected by centrifugation, resuspended, and grown in 20 ml of SL-ura medium overnight at 30°C. These cells were then collected and resuspended in SG-ura medium and grown for 4 hr at 30°C for induction of *GAL1*::*pma1* expression.

The cellular localization of Pma1 was determined first using a monoclonal antibody, F10, directed against an unspecified epitope in Pma1 protein by indirect immunofluorescence (ref. 16; J.H.T., unpublished data). Localization of HA-tagged, galactose-induced Pma1 proteins, either wild type or mutant, was determined by indirect immunofluorescence staining using an anti-HA antibody (Berkeley Antibody, Richmond, CA). Similar indirect immunofluorescent staining was carried out using an anti-Kar2 antibody provided by M. Rose (17).

RESULTS

Introduction of Mutant *pma1* Genes into Yeast. Strain SH122 was transformed with the *Hind*III *PMA1*::*URA3* fragments containing an *in vitro*-generated mutation (Fig. 1*A*). All Ura$^+$ Leu$^-$ transformants must have obtained the mutation. The *pma1* allele containing mutations can then be isolated away from the wild-type *PMA1* gene by sporulation of the diploid and tetrad dissection. The two Ura$^+$ segregants should therefore contain the mutation carried on the transforming fragment. The results for a number of different *in vitro*-generated mutations are presented in Table 1. Recessive mutations such as A135I are viable, but show reduced growth, while A135R and A135D are recessive lethal, yielding only two viable Ura$^-$ segregants per tetrad. The results for A135W and D378N appear to suggest that these alterations are inconsequential, as four wild-type spores are produced (Table 1).

Loss of *pma1-D378N* by Gene Conversion. The tetrad data for D378N seemed to confirm a previous report that D378N is apparently a viable, wild-type substitution (6). However, when we sequenced the DNA prepared from each segregant

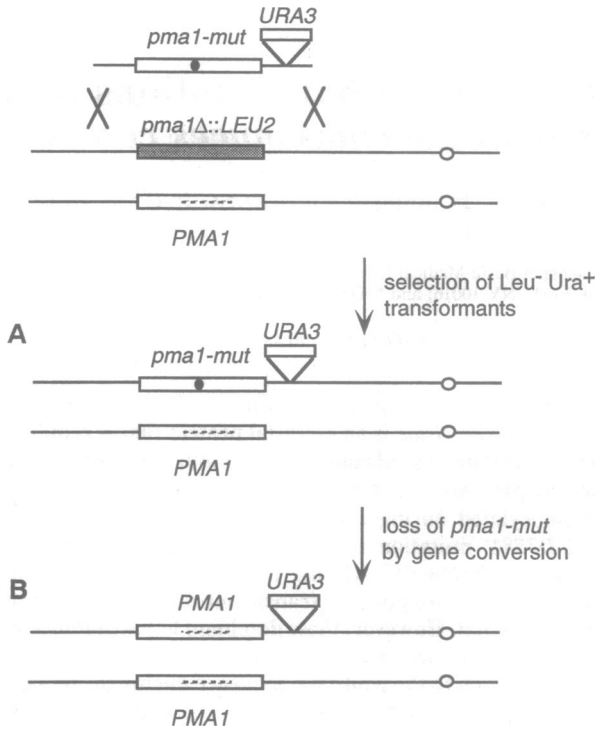

FIG. 1. Introduction of mutant *pma1* sequences and the gene conversion of dominant lethal mutations. (*A*) *PMA1* can be replaced with mutant *pma1* genes by using a modification of the one-step gene replacement technique (18). Because recombination must take place within regions of homology, all Ura$^+$ Leu$^-$ transformants should contain the desired mutation and Ura$^+$ haploid segregants, obtained after meiosis, will reveal the mutant phenotype. (*B*) If the transforming fragment contains a dominant lethal mutation, this mutation can be eliminated by gene conversion between the incoming fragment and the resident, wild-type *PMA1** gene (in this case marked with 12 silent base-pair substitutions). When Ura$^+$ haploid segregants are analyzed, they will appear to be wild type, as the lethal mutation has been lost.

in four tetrads, one each from four different transformants, none of the 16 segregants carried the D378N mutation (data not shown). Apparently, the mutation was lost either by gene conversion involving either *PMA1* or *PMA2* (7, 17, 19) or by base-pair substitution.

To determine how the mutation was being lost, we repeated the transplacement experiment using diploid strain

Table 1. Introduction of *in vitro*-generated mutations into *pma1*Δ::*LEU2*/*PMA1* diploids SH122 and SH129

Transforming DNA	% Ura$^+$ Leu$^-$ transformants*	Phenotype of Ura$^+$ segregants
PMA1	14	Wild type
pma1-S368F	11	Recessive mutant
pma1-A135V	11	Recessive mutant
pma1-A135D	8	Recessive lethal
pma1-A135W	10	Apparently wild type; actually dominant lethal
pma1-D378N	7	Apparently wild type; actually dominant lethal

The transforming DNA carried the designated mutation linked to the *URA3* gene inserted at the 3′ end of the *PMA1* gene, as illustrated in Fig. 1. Phenotypes of haploid, Ura$^+$ meiotic segregants of tetrads from Ura$^+$ Leu$^-$ diploids were analyzed. For each transforming DNA at least five tetrads from two independent Ura$^+$ Leu$^-$ diploid transformants were analyzed.

*At least 80 Ura$^+$ transformants were scored for Leu$^+$. Percentages for *PMA1* and *pma1-D378N* DNA represent combined data for transformations into SH122 and SH129.

SH129 in which the wild-type *PMA1* gene was replaced by the *PMA1** allele. The *PMA1** allele in this strain ([*pma1*Δ::*LEU2*]/*PMA1**) contains 12 silent mutations in the 66 bp surrounding the GAC (D378) codon, resulting from a gene conversion event between *PMA1* and its unexpressed homologous gene, *PMA2* (19). When the *Hin*dIII *pma1-D378N, URA3* fragment was transplaced into strain SH129, all Ura$^+$ segregants from each of 10 independent Ura$^+$ Leu$^-$ diploids were wild type. DNA samples from one Ura$^+$ segregant of each of 10 transformants were amplified by PCR and sequenced. Every one of these new Pma$^+$ alleles contained all 12 silent mutations from the *PMA1** allele (Fig. 1*B*). This clearly demonstrates that the loss of the D378N mutation occurred by gene conversion and not by a simple reversion of a base-pair substitution.

Transient Expression of *pma1-D378N* Causes the Arrest of Cell Growth. A more direct demonstration of the dominant lethal effect of *pma1-D378N* was obtained by transforming both wild-type haploid and diploid strains with a centromere-containing plasmid, pXZ09, that carries the *GAL1*::*pma1-D378N* gene (Fig. 2*A*; see also *Materials and Methods*). When these strains were transferred from glucose- to galactose-containing medium lacking uracil, the cells were unable to grow (Fig. 2*B*). A small number of papillae were observed, most likely representing revertants or gene convertants that had lost the *pma1-D378N* allele. This result is also seen in liquid-grown cultures (data not shown). These results demonstrate directly that the D378N mutation is dominant lethal. Diploid cells carrying the wild-type *PMA1* gene are not as strongly arrested, which suggests that the D378N dominant lethal allele is partially suppressed by a 2-fold change in *PMA1* gene dosage. *pma1-D378N* still exhibits dominant lethalilty when the wild-type protein is also expressed from the same *GAL1* promoter (see below).

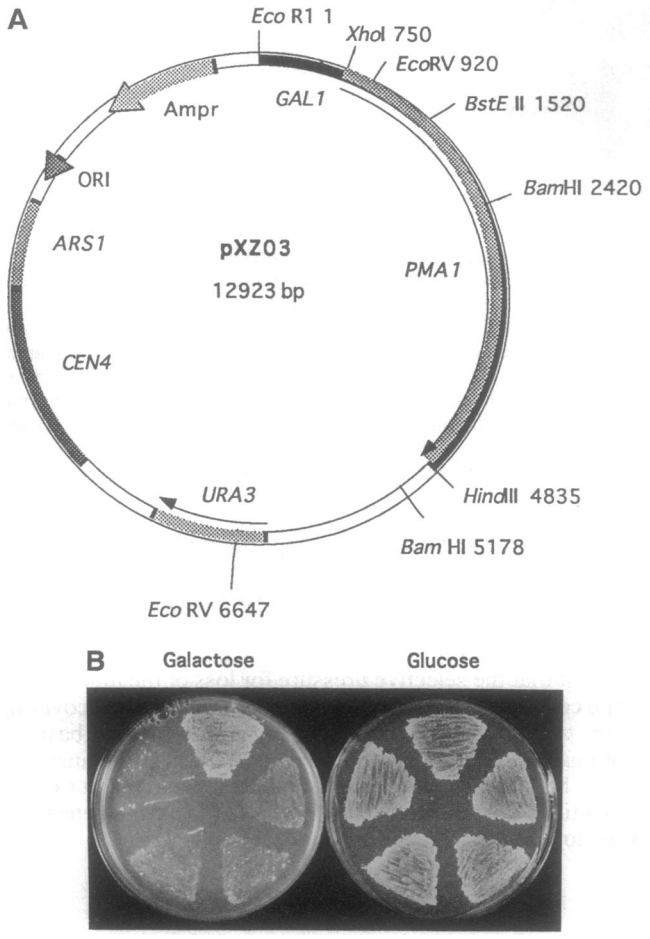

FIG. 2. Demonstration of dominant lethality by expressing a galactose-inducible *pma1* gene carrying a dominant lethal mutation. (*B*) Growth of haploid strains transformed with plasmids carrying a galactose-inducible wild-type or dominant lethal *PMA1* gene and grown on glucose (right) or galactose (left). The patch of cells at the top is transformed with the wild-type *PMA1* gene in pXZ03 (*A*). The other cells, reading clockwise, were transformed with plasmids carrying A135F F144A, A135F F144S, A135W, and D378N, respectively.

Expression of the Dominant Lethal D378N Mutation Causes Accumulation of Pma1 Protein in Discrete Cytoplasmic Structures. There was no characteristic cell-cycle arrest phenotype after galactose induction of *pma1-D378N*, as cells with all sizes of buds were obtained. However, there was a distinctive phenotype associated with this arrest. With cells grown under permissive conditions (glucose or lactate medium), the anti-Pma1 antibody F10 decorates the cell periphery, consistent with the location of the Pma1 protein in the plasma membrane (data not shown). However, cells shifted to galactose medium show a significant accumulation of Pma1 in a set of discrete cytoplasmic structures (Fig. 3*B*). Such structures are not seen in galactose-grown cells carrying the control plasmid pXZ03, which contains an inducible wild-type *PMA1* gene (Fig. 3*A*). The location of these subplasma membrane structures is distinct from the location of the DAPI-stained nucleus. These structures are highly reminiscent of those recently observed after overexpression of the normally unexpressed homologue, Pma2 (20). These same immunofluorescent dots are seen in all of the other dominant lethal mutations we describe below. One other example, for *pma1-A135W*, is shown in Fig. 3*C*.

Because these strains express wild-type Pma1 protein (already in the plasma membrane before induction) and the galactose-inducible gene, it is not possible with immunofluorescent straining of all Pma1 protein to determine if the dominant lethal protein is accumulating only in the unusual submembrane bodies or if it is also capable of being transported to the cell surface. To answer these questions we added a 9-amino acid HA epitope to the N terminus of the Pma1 protein (plasmid pXZ28). The D378N mutation was introduced into pXZ28, as described above, to create pXZ29. When the wild-type, HA-tagged gene was expressed for 4 hr and stained with anti-HA antibody, the tagged wild-type protein was located only at the surface of the cells, as expected (Fig. 3*D*). In contrast, when the HA-tagged, D378N-containing protein was expressed for 4 hr, it was not visible at the plasma membrane surface, but only in the novel cytoplasmic dots (Fig. 3*E*).

The trapping of the mutant Pma1 protein could reflect its inability to exit some part of the secretory apparatus during its normally slow (1 hr) transit to the plasma membrane (21). However, we find that its effect is more global, in that wild-type PMA1 protein also becomes trapped in the cytoplasm and fails to appear at the cell surface. This was demonstrated by placing two centromere plasmids into yeast: *URA3*-marked pXZ09 carrying the galactose inducible *pma1-D378N* gene and *LEU2*-marked pXZ33 with a galactose-inducible wild-type *PMA1* gene carrying the HA epitope. When HA-tagged wild-type protein was induced at the same time as the dominant lethal protein, the anti-HA antibody only stained the cytoplasmic dots characteristic of the mutants (Fig. 3*F*).

It appears these cytoplasmic bodies derive from—or at least contain proteins that are normally associated with—the endoplasmic reticulum (ER). This is evident from the staining of cells with an antibody directed against the chaperone protein, Kar2. As reported previously (17), Kar2 is normally localized in ER surrounding the (DAPI-stained) nucleus (Fig. 3*G*). In contrast, when *pma1-D378N* is expressed, much of

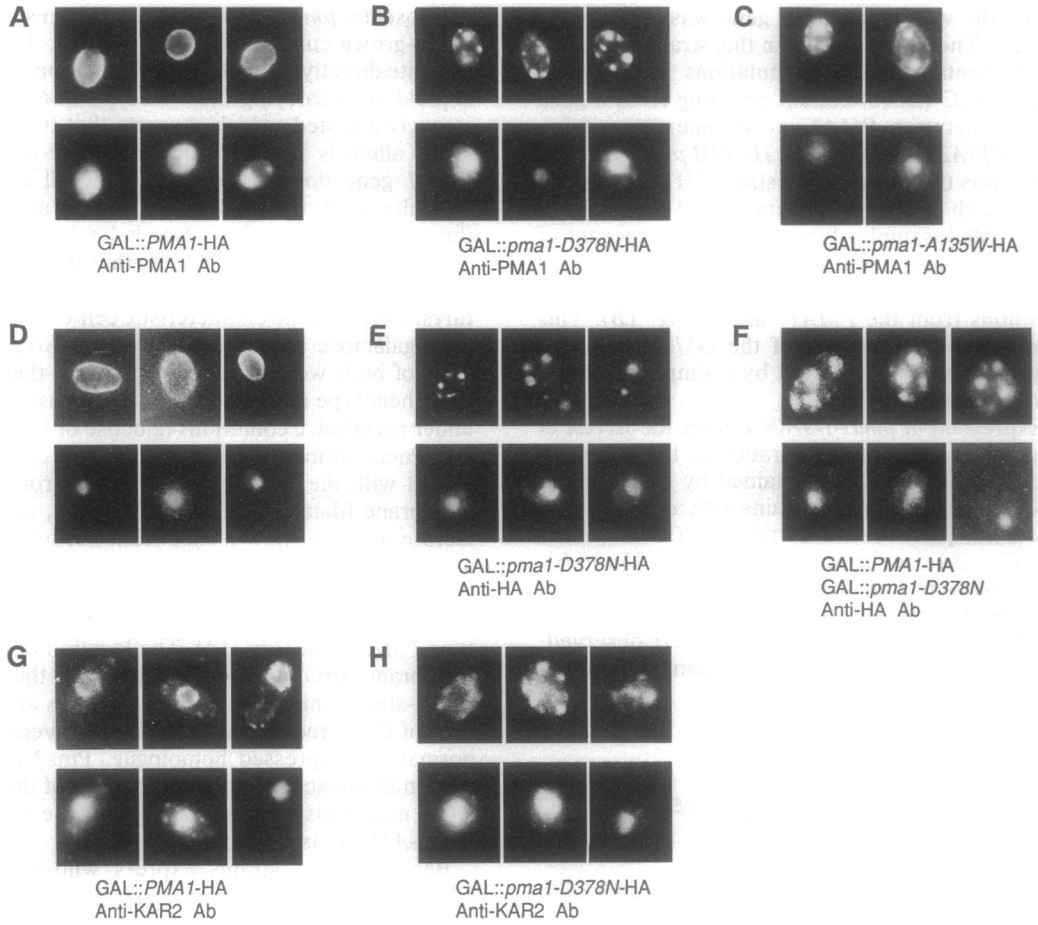

FIG. 3. Accumulation of cytoplasmic structures in cells expressing dominant lethal *pma1*- mutations. Cells carrying either pXZ03 (*PMA1*), pXZ09 (*pma1-D378N*), or pSN122 (*pma1-A135W*) were induced with galactose for 4 hr. In each panel, indirect immunofluorescence using one of three antibodies is shown. The lower panel shows 4',6-diamidino-2-phenylindole (DAPI) staining of the nucleus. In cells expressing only wild-type, HA-tagged *PMA1*, immunofluorescence using an anti-Pma1 antibody is found only around the cell periphery, as expected for a plasma membrane protein (*A*). When similar cells are stained with anti-HA antibody (*D*), the same pattern is observed. When these cells are stained with an anti-Kar2 antibody, bright perinuclear staining is observed (*G*). In cells expressing the dominant lethal mutation, there is staining of the periphery and of several large cytoplasmic structures (*B*). When the similar cells are stained with anti-HA antibody, only the cytoplasmic structures are stained (*E*), indicating that the mutant protein does not get transported to the plasma membrane. When these cells are stained with anti-Kar2 antibody, the immunofluorescence is seen around the nucleus and in highly concentrated "dots" (*H*) similar to that observed with anti-HA antibody. Similar staining of cytoplasmic dots is seen when the dominant lethal mutation *pma1-A135W* is expressed and stained with anti-PMA1 antibody (*C*).

the anti-Kar2 staining is found in dots similar to those observed for the mutant *pma1-D378N* protein (Fig. 3*H*).

Dominant Negative Mutations Are Also Found in the Region of the First Two Transmembrane Domains. The first two transmembrane helices of *PMA1* (H1/H2) comprise a domain that appears to be tightly conformationally coupled to the active site (7, 17). Several site-directed mutations in this region are also dominant lethal. As with D378N, when the *pma1* mutation A135W was transformed into SH122 all four segregants were wild type and all Ura$^+$ segregants had lost the original mutations (data not shown). *pma1-A135W* also proved to be dominant lethal when expressed from the galactose-inducible promoter (Fig. 2). Dominant lethality has also been demonstrated for several double mutant pairs in the H1/H2 region: A135V F144W, A135V F144Y, A135V F144V, A135F F144G, and A135V F144A (Fig. 2 and data not shown). In these cases, the single mutations of A135 and F144 are recessive, and viable, but the double mutants are dominant lethal. Several of the double mutations were not always gene converted away when Leu$^+$ Ura$^-$ transformants were sporulated and dissected. The A135F F144A mutation, which is clearly dominant lethal (Fig. 2), was lost in only 12 of 25 transformants. However, the loss was always all-or-none—that is, all cells of a particular transformant gave rise only to four wild-type segregants in which the mutation was lost or else only yielded two Ura$^-$ wild-type segregants and two slow-growing Ura$^+$ segregants characteristic of a recessive defect. This suggests that the loss of the mutation occurred before the initially transformed cell had divided and may suggest that the selective pressure for loss of the mutation by gene conversion is especially acute when cells are recovering from transformation. This also indicates that the basis of dominant lethality for alterations in the first two transmembrane helices may not be the same as for a knockout of the essential D378 residue, which was lost in 25 of 25 independent transformants.

DISCUSSION

We have discovered that a number of different mutations of the yeast H$^+$-ATPase gene are dominant lethal. These mutations are lost through gene conversion with a wild-type *PMA1* gene during the time that both genes are present and expressed in the same cell. We show that these mutations were indeed dominant lethal by using a conditionally expressed promoter. Cells expressing dominant lethal alleles lead to the accumulation of mutant and wild-type Pma1 protein in a small number of cytoplasmic structures that stain

intensely with anti-Pma1 antibodies. These structures appear to be derived from the ER as they also stain with antibodies directed against the ER protein Kar2.

Finding that D378N is dominant lethal agrees with studies of the mammalian Ca^{2+}-ATPase, where the cognate mutation is essential (5). Whether the mammalian mutant is also dominant lethal is not known. We have also found that several changes in the putative first two transmembrane helices of the protein are dominant lethal. All seven of the dominant lethal mutations we have examined cytologically share the same arrest phenotype, with the accumulation of Pma1 in a few intensely staining cytoplasmic structures.

A mutant protein might be dominant negative by competing with the wild type for substrate molecules. With multimeric proteins, the mutant protein may associate with wild type to produce an inactive oligomeric complex. A third possibility is that the mutant Pma1 protein might be transported to the plasma membrane but then create unregulated ion channels that could collapse the cell's membrane potential. Alternatively, the mutant ATPase might enter the secretory apparatus and fail to be properly transported, thus "clogging" the secretion pathway and arresting cell growth. This could occur either because the mutant protein was misfolded or if the mutant protein depolarized the ER or other compartments of the secretory apparatus.

The fact that mutant Pma1 protein is not transported to the surface makes unlikely the idea that the dominant lethality is due to some failure of the ATPase on the plasma membrane. Moreover, since *pma1-D378N* is still dominant lethal when two other copies of *PMA1* are expressed (one from the same galactose-driven promoter), it is also unlikely that the mutant proteins are poisoning the function of a multimeric plasma membrane H^+-ATPase. These results seem most consistent with the idea that the dominant mutant proteins interfere with transport of wild-type H^+-ATPase and possibly other proteins. The fact that Kar2 is found in such cytoplasmic structures suggests that these structures are derived from the ER, though the possibility that the Kar2 (BiP) chaperone protein might have been carried into another compartment cannot be discounted.

Recently, similar unusual cytoplasmic structures that appear to derive from the ER were observed when the normally unexpressed *Saccharomyces* Pma2 protein is transcribed from a *PMA1* promoter on a high-copy plasmid (20). Expression of the *Arabidopsis* H^+-ATPase homologue also leads to its accumulation in ER-like membranes, but not in the plasma membrane (22). However, the accumulation of these heterologous Pma1 proteins in the cytoplasm is apparently not dominant lethal. Mutations preventing H^+-ATPase transport to the plasma membrane have also been observed by Rao and Slayman (23). They recently showed that six different glycine insertions at the phosphorylation domain (amino acids 378–383) all failed to be accumulated in the secretory vesicles even though the 100-kDa polypeptide was synthesized and was relatively stable. We do not know if these mutants would show the same sort of cytoplasmic structures we have observed by immunofluorescence. Coupled with our demonstration, and that of the Goffeau laboratory (an unpublished result mentioned in ref. 20), that the Kar2 protein also accumulates in these unusual structures, it seems likely that they derive from the ER rather than from later stages of secretion.

Our findings have important implications for studies of the *PMA1* gene and for analogous *in vitro* mutagenesis studies of other essential genes in yeast. All sequence shuffling paradigms require that the mutant gene be coresident in the cell with a wild-type gene for some time before the wild-type gene on a plasmid is evicted from the cell or a wild-type gene on a chromosome is either turned off or segregated away (24–27). During this time, a dominant lethal mutation can be replaced by gene conversion with the wild-type allele. Thus, it is essential to confirm that any apparently harmless mutation is actually present after sequence shuffling.

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