J. Biochem. 129, 827–833 (2001)

Characterization of a *Saccharomyces cerevisiae* Gene That Encodes a Mitochondrial Phosphate Transporter–Like Protein[1]

Reona Takabatake,[*,2] Abu-Baker M. Siddique,[‡,2,3] Hiroshi Kouchi,[‡] Katsura Izui,[*,†] and Shingo Hata[*,†,4]

[*]Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502; [†]Laboratory of Plant Physiology, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8502; and [‡]National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305-8602

Received December 28, 2000; accepted March 7, 2001

The mitochondrial phosphate transporter of *Saccharomyces cerevisiae*, encoded by *MIR1* (*YJR077C*) gene, shows divergence among the transporters in various eukaryotes. We have characterized another gene, *YER053C*, that appeared to encode an orthologous mitochondrial phosphate transporter of yeast. The predicted amino acid sequence of the YER053C protein is much more similar to that of mitochondrial phosphate transporters of other species than that of MIR1. RNA gel blot analysis indicated that, like the *MIR1* promoter, the *YER053C* promoter is functional and that its activity varies according to aeration. An *MIR1* gene null mutant did not grow on glycerol medium, whereas a *YER-053C* null mutant grew well on the medium, suggesting that the *YER053C* gene is not essential for the mitochondrial function. *YER053C* also did not support the growth of the *MIR1* null mutant on glycerol. The MIR1 and YER053C proteins were expressed in *Escherichia coli* and then reconstituted into liposomes. Unlike the proteoliposomes of MIR1, those of YER053C did not exhibit significant phosphate transport activity. Unexpectedly, it was shown that YER053C is localized in vacuoles, not mitochondria, by immunological electron microscopy. These results suggest that, during evolution, yeast lost the function and/or mitochondrial targeting of YER053C and then recruited an atypical MIR1 as the only transporter.

Key words: mitochondrial phosphatetransporter, paralog, pseudogene, vacuole, yeast.

The transport of Pi into the mitochondrial matrix is essential for the oxidative phosphorylation of ADP to ATP. The mitochondrial phosphate transporter (MPT) responsible for this transport is located in the inner membrane. MPT forms a homodimer, and catalyzes P_i/H^+ symport or P_i/OH^- antiport as well as P_i/P_i exchange (*1–5*). The molecular cloning of MPT genes has been achieved from animals (*6–10*), plants (*11, 12*), and yeasts (*13, 14*; DNA database accession number, AL136536). The MPTs of both animals and plants are sensitive to *N*-ethylmaleimide (NEM) because they have a common NEM-reactive Cys residue (Cys42 for beef heart MPT) (*6–8, 11, 12*). This Cys residue was once suggested to be essential for the P_i transport activity (*15, 16*). However, Wohlrab and coworkers revealed that the MPT of yeast *Saccharomyces cerevisiae* is unique in that (i) it is insensitive to NEM (*17*), (ii) the NEM-reactive Cys residue is replaced with Thr (*14*), and (iii) its peptide map is different from that of beef heart MPT (*17*). Here we refer to the gene (YJR077C) that encodes the well-characterized MPT in *S. cerevisiae* as *MIR1*. The gene product, the MIR1 protein, was once reported to be a receptor for protein im-port into mitochondria (*13, 18, 19*). However, recent progress in research on yeast MPT has revealed that this is unlikely (*4, 5, 20, 21*).

During a database search for transporters similar to plant MPTs (*12*), we unexpectedly found that there is another gene, *YER053C*, for an MPT-like protein in the *S. cerevisiae* genome. The finding that the YER053C proteincontains a putative NEM-reactive Cys residue prompted us to examine whether or not YER053C is involved in the P_i transport by yeast mitochondria. In the present investigation, we isolated both *MIR1* and *YER053C* with the aid of PCR, examined their mRNA levels, and determined their transport activities *in vitro* after expression of recombinant MIR1 and YER053C in *Escherichia coli* and reconstitution with liposomes. We disrupted each of *MIR1* and *YER053C* by homologous recombination and characterized the resulting null mutants. We also examined the subcellular localization and stability of the YER053C protein. The molecular evolution of the two yeast genes is discussed.

MATERIALS AND METHODS

Strains and Plasmids—*S. cerevisiae* INVSc1 (*MATα, his3Δ1, leu2, trp1-289, ura3-52*) was purchased from Invitrogen (Carlsbad, CA) and used as a wild-type strain

[1] This work was supported in part by the Special Coordination Funds of the Science and Technology Agency of the Japanese Government.

[2] These authors contributed equally to this work.

[3] Present address: Department of Botany, Jahangirnagar University, Savar, Bangladesh.

[4] To whom reprint requests should be addressed. E-mail: shing@kais.kyoto-u.ac.jp

Abbreviations: MPT, mitochondrial phosphate transporter; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; YPD, yeast extract/peptone/glucose; YPG, yeast extract/peptone/glycerol.

throughout this study. Another wild-type strain M10 (*22*) was used only to isolate genomic DNA and to amplify the *HIS3* gene by PCR. The yeast expression vector pYES2 was also obtained from Invitrogen. Cells were transformed by the lithium acetate method (*23*).

Growth of Yeast Cells—Yeast cells were grown aerobically at 28°C in yeast extract/peptone/glucose (YPD) medium or yeast extract/peptone/glycerol (YPG) medium, or under selective conditions in SC medium supplemented with nutritional requirements as necessary (*24*). Cells were also grown anaerobically in YPD medium containing 5 μg/ml cholesterol and 15 μl/ml Tween 80. Under anaerobic conditions, the oxygen concentration was kept below 0.1% by means of an Anaeropack (Mitsubishi Gas Chemicals, Tokyo). Synthetic minimal medium was prepared according to Burkholder (*25*). P_i-free and low-P_i media were prepared by the substitution of KCl for KH_2PO_4 in the minimal medium.

Gene Cloning, Disruption, and Complementation Experiments—*YER053C* was amplified from INVSc1 DNA by PCR with the following primer pairs: for the entire coding region, 5′-GTTCTAGACCATGGAGTCCAATAAACAACC-3′ and 5′-ATCCTCGAGCTAACCGGTGGTTGGTAAG-3′; and for the 5′-noncoding region, 5′-TTGAGCTCTCAGCACATAGGTAATAAGGT-3′ and 5′-GCTCTAGAACTGTCAATGAGTCCGTTCTC-3′. *MIR1* was also amplified from INVSc1 DNA with: for the entire coding region, 5′-TCTCTAGACCATGGCTGTGTCTGCTGCTCCTGC-3′ and 5′-AGACTCGAGCTAATGACCACCACCACCAA-3′; and for the 5′-noncoding region, 5′-TTGAGCTCGGAGCGACGTTGAAAAGTGAC-3′ and 5′-TGTCTAGATGAGACTTTTTGATCTTTGTA-3′. *HIS3* was amplified from M10 DNA with 5′-GCTCTAGACAGGGAAGTCATAACACAGTCC-3′ and 5′-CGAATTCCCGTTCCTCCATCTCTTTTAT-3′. Appropriate restriction enzyme sites were included in the primers to facilitate cloning and construction. DNA sequencing confirmed that the PCR products were exactly the expected ones. *YER053C* was disrupted by the replacement of the 5′ half of the coding region (from the initiation codon to the *Eco*RI site, 360 bp in size) with the *HIS3* gene. *MIR1* was similarly disrupted with *HIS3*, taking advantage of the presence of an *Eco*RI site in the middle of the coding region. Correctly targeted insertion was confirmed by PCR in each case. For complementation experiments, the entire coding region of *YER053C* was ligated downstream of the 5′-noncoding region of *MIR1*, then inserted between the *Sac*I and *Xho*I sites of pYES2. From the resulting plasmid, *YER053C* was transcribed under the control of *MIR1* promoter. The 5′-noncoding and entire coding regions of *MIR1* were also subcloned into pYES2.

Blot-Hybridization Analyses of RNA and DNA—Total RNA and genomic DNA were isolated from yeast as described (*26, 27*). For RNA blot-hybridization analysis, 20 μg of total RNA per lane was fractionated by electrophoresis in a 1% denaturing agarose gel and then blotted onto a Hybond-N^+ membrane (Amersham Pharmacia, Pisscataway, NJ). For DNA blot-hybridization analysis, 10 μg of yeast genomic DNA was digested with *Kpn*I, *Hin*dIII, or *Xba*I. The DNA was separated by 0.8% agarose gel electrophoresis and then blotted onto a Hybond-N^+ membrane. Hybridization with the ^{32}P-labeled entire coding regions of *MIR1* and *YER053C*, and washing of the membranes were performed under high-stringency conditions (*28*).

Pi Transport Assay In Vitro—The MIR1 and YER053C proteins were expressed as inclusion bodies in *E. coli* using the expression vector pET-24d (Novagen, Madison, WI) (*12*). The inclusion bodies were solubilized in a medium (10 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 1 mM dithiothreitol) containing 1.2% (w/v) Sarkosyl. The presence of the solubilized MIR1 and YER053C proteins was confirmed by SDS–polyacrylamide gel electrophoresis (*29*). The solubilized MIR1 and YER053C proteins were reconstituted into liposomes by the freeze-thaw technique (*30*). The phosphate transport reaction was initiated by adding 50 μl (5 kBq) of $^{32}P_i$ to 150 μl of proteoliposomes. After incubation at 25°C, pyridoxal 5′-phosphate and mersalyl (final concentrations, 45 mM and 8 mM, respectively) were added to stop the transport. The mixture was passed through an AG1-X8 column (Bio-Rad, Hercules, CA) with 200 mM sodium acetate to remove unincorporated $^{32}P_i$. The uptake of $^{32}P_i$ was determined by liquid scintillation counting of Cerenkov radiation.

Generation of Tagged YER053C and MRI1—A 3′ portion of the *YER053C* coding region was amplified from INVSc1 DNA by PCR with 5′-CTGAGCTCCTCGCTGATATCATGTTGTGCC-3′ and 5′-GCTCTAGACTACTTGTCATCGTCATCCTTGTAGTCACCGGTGGTTGGTAAGCCTACAT-3′. The latter primer contained the antisense sequences for a FLAG tag and an artificial stop codon so that the tag is fused to the C-terminus of the YER053C protein. The 3′-noncoding region of *YER053C* was amplified with 5′-TGAATTCAATCGGCAAGGTTTATCGTA-3′ and 5′-ATGGGCCCGAAAAATACACGTGGTTAGAGCCA-3′. The PCR products were attached to both ends of *HIS3*, then introduced into wild-type INVSc1 cells to insert the gene for the FLAG epitope and *HIS3* marker around the stop codon of *YER053C*. Similarly, the MIR1 protein was also tagged with the FLAG epitope at the C-terminus by using the following primer pairs: for a 3′ portion of the coding region, 5′-CCGAGCTCTTGGCAGACATTGCCTTGTG-3′ and 5′-CATCGCTCTAGACTACTTGTCATCGTCTTGTAGTCATGACCAC CACCACCAATTTC-3′; and for the 3′-noncoding region, 5′-TGAATTCTCTCCTCACACCACCGGAGC-3′ and 5′-AGATCGGGTCGCCAATACGTGG-3′.

Immunoelectron Microscopy—Yeast cells were cultured in YPG at 30°C overnight, collected by centrifugation, washed with potassium phosphate buffer (50 mM, pH 6.8), and then resuspended in a fixative (2% gluteraldehyde) for 2 h. The fixed cells were washed again with the buffer and dehydrated in ethanol series (30–100%), followed by infiltration and embedding in LR White resin as described by Preuss *et al.* (*31*). At the end of each step, the cells were resuspended and pelleted down by brief centrifugation in an Eppendorf tube. Except for the embedding, all steps were carried out at 4°C. Ultra-thin sections were cut using a diamond knife and mounted on formver-coated 300-mesh nickel grids. Immunostaining was carried out according to the protocol of Ueno (*32*). The grids were incubated for 30 min in ablocking solution of 0.5% (w/v) BSA in phosphate-buffered saline (PBS), then in 100-fold diluted anti-FLAG M2 monoclonal antibodies (Sigma, St. Louis, MO) for 3 h. The grids were then washed several times with PBS and incubated in 40-fold diluted 15-nm Protein A-colloidal gold (E. Y. Lab., San Mateo, CA) for 30 min. After several washes with PBS and a final wash with distilled water, thegrids were post-stained, then examined under a Hitachi H-

7100 transmission electron microscope (Hitachi, Tokyo) at 100 kV. All steps of immunostaining were performed at room temperature.

RESULTS

Relationship of YER053C to the MPT Proteins of Various Species—YER053C was unexpectedly found on a database search for amino acid sequences similar to that of a plant MPT (*12*). As the first step to characterize the gene, both the *MIR1* and *YER053C* genes were amplified by PCR. Since an *Nco*I site was introduced into the initiation codon, the second codon of *MIR1* was changed from TCT to GCT, resulting in the replacement of Ser with Ala. The effect of the replacement should be minimal, because this amino acid is at the N-terminus. Otherwise, the nucleotide sequences of their coding regions were exactly the same as reported (DNA database accession number, U18796; *14*). *MIR1* and *YER053C* encode 311– and 300–amino acid polypeptides with calculated molecular masses of 32.8 and 33.5 kDa, respectively. Interestingly, as shown in Fig. 1, the deduced amino acid sequence of the YER053C protein is more

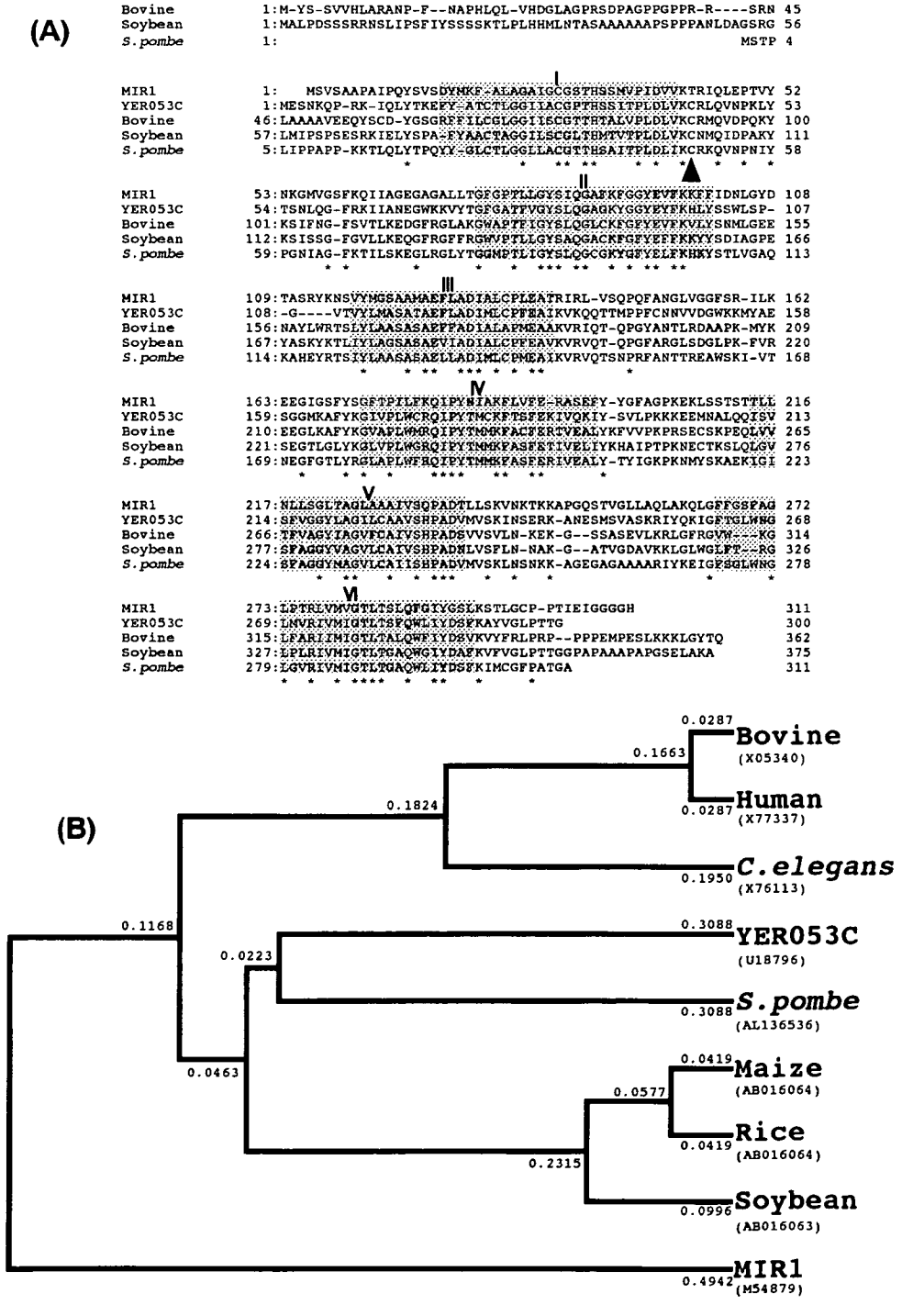

Fig. 1. **Alignment of and phylogenetic relationship among MIR1, YER053C, and MPTs of other species.** (A) The deduced amino acid sequences of *MIR1* and *YER053C* were aligned with those of bovine MPT, soybean MPT, and *Schizosaccharomyces pombe* MPT. Dashes denote gaps introduced to facilitate the alignment. Asterisks indicate residues identical throughout. The potential transmembrane domains are shaded and denoted by Roman numerals. The predicted NEM-reactive Cys residue is indicated by an arrowhead. (B) Phylogenetic tree for YER053C and MPTs. The unweighted pair group method with an arithmetic mean tree (*50*) was used for the polypeptides, using Genetix-Mac software (Software Development, Tokyo). The values near the branches give the branch lengths in the numbers of amino acid substitutions per site. The database accession numbers are indicated in parentheses.

typical than that of MIR1 among the MPTs so far characterized. Like MIR1, YER053C does not have an apparent signal peptide for mitochondrial targeting at its N-terminus. The predicted hydropathy plot of YER053C with six membrane-spanning segments was also very similar to those of other MPTs (data not shown). More strikingly, the YER053C protein has a putative NEM-reactive Cys residue at the 44th position from the N-terminus between positively charged amino acids. A protein database search using the amino acid sequence of YER053C as a probe only revealed MPTs of other species with high scores. Therefore, YER053C seemed at first sight to be an orthologous P_i tranporter, whereas MIR1 was somewhat unusual.

Expression of YER053C Transcripts—Restriction enzyme-digested genomic DNA of the wild-type *S. cerevisiae* was subjected to Southern blot analysis using the coding regions of *MIR1* and *YER053C* as probes. Each gene was detected as a single band and did not cross-hybridize with the other under the conditions (data not shown). This result is consistent with the descriptions in databases that there is only one copy each in the yeast genome; *MIR1* lies in chromosome X, whereas *YER053C* is in chromosome V. As shown in Fig. 2, A and B, the mRNA levels of both *YER053C* and *MIR1* were higher in aerobically-grown than in anaerobically-grown wild-type cells. Thus, the promoters of both *YER053C* and *MIR1* are functional and respond to aeration.

YER053C Is Not Essential for Mitochondrial Oxidative Phosphorylation—The 5′ portion (around 40% of the coding region) of *YER053C* was replaced with *HIS3*, resulting in a null mutant, *yer053c::HIS3*. *MIR1* was also disrupted by *HIS3* in a similar way, producing another mutant, *mir1::HIS3*. The mutants did not produce normal transcripts for MPTs, confirming the gene disruption. However, they contained unusually short transcripts (Fig. 2, C and D). The short transcripts could not produce normal proteins because significant portions of their coding regions were removed. There may be some promoter activity within the *HIS3* region. Yeast growth on plates with glycerol, a nonfermentable carbon source, has been considered as an indication of physiological competence of MPTs (*33, 34*). As shown in Fig. 3A, *mir1::HIS3* did not grow on glycerol, con-

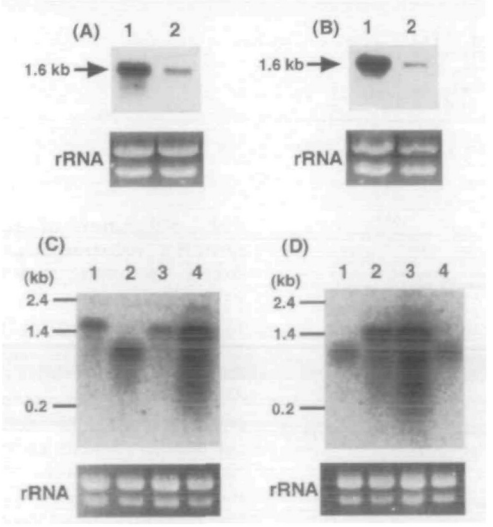

Fig. 2. **Expression of the *YER053C* and *MIR1* transcripts of wild-type and mutant yeast cells under various conditions.** (A and B) Blot-hybridization analysis of RNAs extracted from wild-type cells. The membrane was probed with the coding region of *YER053C* (A) or *MIR1* (B). Lane 1, aerobically-grown cells; and lane 2,anaerobically-grown cells. (C and D) RNAs were extracted from aerobically grown cells in YPD medium. Lane 1, *mir1::HIS3*; lane 2, *yer053c:: HIS3*; lane 3, *mir1::HIS3* transformed with pYES2 harboring *MIR1*; and lane 4, *mir1::HIS3* transformed with pYES2 harboring *YER053C*. Ten microgram of total RNA was fractionated, blotted onto a membrane, and then probed with the coding region of *YER053C* (C) or *MIR1* (D). The normal transcripts of both *YER053C* and *MIR1* were 1.6 kb in size, whereas the shorter transcripts were 0.8 kb in size. The lower panels show the ethidium bromide-stained rRNAs in the gels.

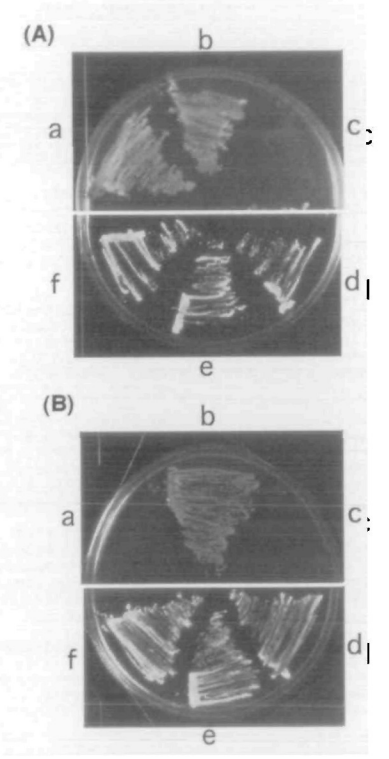

Fig. 3. **Growth of yeast cells on glycerol (YPG) or glucose (YPD).** (A) Wild-type and mutant cells which had been precultured on YPD plates were streaked onto YPG plates and then grown at 30°C for 3 d (a–c) or on YPD plates and then grown at 30°C for 2 d (d–f). a and d, *yer053c::HIS3*; b and e, wild type; and c and f, *mir1::HIS3*. (B) *mir1::HIS3* was transformed with the pYES2 constructs, then grown as in A. a and d, transformant with the *MIR1* promoter-*YER053C* coding region; b and e, transformant with the *MIR1* promoter-*MIR1* coding region; and c and f, vector control.

TABLE I. **The uptake of $^{32}P_i$ by proteoliposomes containing recombinant MIR1 or YER053C.** The P_i/P_i exchange activity was assayed as described (*12*). Means ± SE for three independent experiments.

	Incubation (min)	MIR1 (nmol/mg protein)	YER053C (nmol/mg protein)
Exp. 1[a]	10	17.1 ± 3.0	3.3 ± 0.7
	30	17.9 ± 2.5	3.8 ± 0.5
Exp. 2[b]	10	16.6 ± 3.7	2.8 ± 0.8
	30	17.4 ± 2.3	2.0 ± 0.7

[a]The net uptake of ^{32}P was calculated by subtracting the uptake into control liposomes without proteins, in which 0.5 mM P_i was present. [b]The internal P_i of the proteoliposomes and control liposomes was replaced with 0.5 mM KCl.

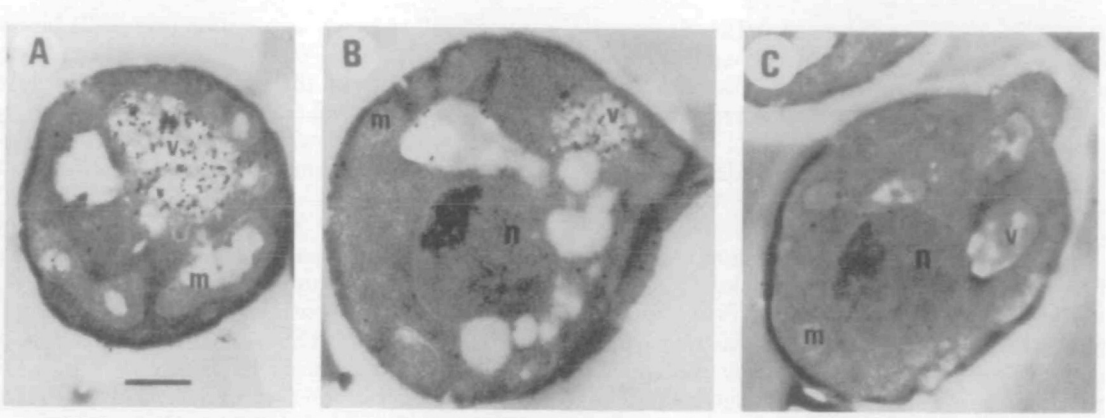

Fig. 4. **Immunogold detection of YER053C tagged with a FLAG epitope.** (A and B) Yeast cells expressing the tagged YER053C. (C) Wild-type cells. v, vacuoles; m, mitochondria; and n, nuclei. Bar, 1 μm.

firming the previous reports (*13, 33, 34*). In contrast, *yer053c:HIS3* grew well on the glycerol medium (Fig. 3A). Apparently, the *YER053C* disruption had no effect on the mitochondrial function. These results are in accord with previous observations (*13, 34–37*) and indicate that MIR1 is essential for oxidative phosphorylation.

YER053C Cannot Substitute for MIR1—In the initial stage of this work, we were not sure whether the YER053C protein is expressed in yeast cells. Therefore, we constructed an expression vector for yeast by inserting thecoding region of *YER053C* just downstream of the 5′-noncoding region of *MIR1*. Since the 5′-noncoding region contains not only the *MIR1* promoter but also a ribosome binding site, the YER053C protein should be generated under the control mechanism for MIR1 production. As a positive control, the coding region of *MIR1* was also connected to its own promoter. We then transformed the null mutant *mir1::HIS3* with the recombinant genes. The exogenous genes were highly expressed due to a gene-dosage effect (Fig. 2, C and D). As shown in Fig. 3B, exogenous *MIR1* supported the growth of *mir1::HIS3* on glycerol. On the other hand, exogenous *YER053C* did not support its growth even under the control of the *MIR1* promoter.

YER053C Is Inactive in Pi Transport In Vitro—The entire coding regions of *MIR1* and *YER053C* were inserted into the pET24d plasmid, such that each gene was expressed under the control of the T7 *lac* promoter. The vectors were introduced into *E. coli*. Both recombinant proteins, around 33 kDa in size, were expressed as inclusion bodies and then solubilized. The purity of the preparations seemed to be more than 80% (data not shown). The recombinant proteins were reconstituted with liposomes. Ultracentrifugation of the resulting proteoliposomes and subsequent SDS-PAGE of the precipitates indicated that nearly the same amounts of MIR1 and YER053C were incorporated into the liposomes (data notshown). As shown in Table I, the MIR1 proteoliposomes exhibited high P_i-transport activity. The uptake reached equilibrium within 10 min. In contrast, the uptake of P_i into the YER053C proteoliposomes was very low, if any, even after 30 min of incubation.

YER053C Is Targeted to Vacuoles—The subcellular localization of YER053C was examined by electron microscopy. Tagging at the C-terminus of YER053C with a FLAG epitope and immunogold detection with anti–FLAG M2 antibodies revealed that YER053C is present in vacuoles (Fig. 4A). The immunogold signals were dispersed within vacuoles, not localized on membranes. Notably, no signals were detected in mitochondria (Fig. 4, A and B). YER053C was also not located in nuclei (Fig. 4B). Vacuoles in the wild-type yeast without the FLAG epitope did not give positive signals (Fig. 4C). It was also indicated that the *YER053C* transcript is translatable in yeast cells.

DISCUSSION

In the present study, we examined the expression of *YER053C* and characterized the MPT-like protein product, YER053C, in *S. cerevisiae*, a very popular model eukaryote. It is known that MPTs form a subfamily of the mitochondrial transporter family (*38*). The phylogenetic relationship of YER053C to the MPTs of other species was unclear, though it has been pointed out that YER053C is closely related to MIR1 among yeast transporters (*36, 39*). *YER053C* seemed at first sight to encode an orthologous P_i transporter among the family members (Fig. 1). Although the activities of the *MIR1* and *YER053C* promoters under various respiratory conditions have been studied with the aid of DNA microarrays, the results were very controversial (*40–42*). We obtained the first unequivocal evidence that both *YER053C* and *MIR1* promoters are activated in response to aerobicity (Fig. 2). We also demonstrated that the *YER053C* transcript is translatable (Fig. 4), like that of *MIR1*. However, the intact YER053C protein does not exhibit transport activity *in vitro* (Table I). More strikingly, YER053C is localized invacuoles (Fig. 4). It has been reported that the FLAG epitope does not affect protein sorting in yeast cells (*43*). Thus, MIR1 is the only functional mitochondrial P_i transporter in *S. cerevisiae*. Experiments with null mutants of these genes supported this idea (Fig. 3).

When a preliminary protein gel blot analysis was conducted to check the integrity of the proteins, a clear band of the epitope-tagged MIR1, about 33 kDa in size, was detected in the membrane fraction (unpublished data). In sharp contrast, the epitope-tagged YER053C gave no distinct bands in either soluble fraction or membrane fraction (unpublished data). This is probably because a large portion of YER053C has been degraded. The immunogold signals detected in vacuoles at a high level (Fig. 4) seem to derive from remnants of the YER053C protein.

When Guerin *et al.* (*17*) solubilized MIR1 from yeast mitochondria, they copurified the ADP/ATP carrier, another member of the mitochondrial transporter family, but did not detect YER053C. If intact YER053C is present in mitochondria, it should be copurified with MIR1, because it is much more similar to MIR1 than is the ADP/ATPcarrier. The localization and degradation of YER053C in vacuoles (Fig. 4 and unpublished data) solved this problem.

At first, we could not understand why immunogold signals of YER053C are dispersed in vacuoles (Fig. 4), although the protein has six putative transmembrane segments (Fig. 1). Then, however, YER053C was suggested to be undergoing proteolysis probably for amino acid recycling (unpublished data). It is noteworthy that high-affinity hexose transporters Hxt6 and Hxt7 of *S. cerevisiae* are functional when cells are grown in a low glucose medium, but if a high concentration of glucose is added to the medium, they are rapidly degraded in vacuoles (*44*). By analogy, we hypothesized that YER053C may play a role under low-P_i conditions, because YPD and YPG are P_i-rich media. We compared the growth rates of the wild-type, *mir1::HIS3* and *yer053c::HIS3* cells in synthetic minimal media containing various concentrations of P_i (0 μM, 1 μM, 10 μM, 0.1 mM, and 10 mM). The growth rates were dependent on the P_i concentrations. However, no significant difference was detected among the wild-type, *mir1::HIS3* and *yer053c::HIS3* under the low-P_i conditions (unpublished data). At present, we cannot completely exclude the possibility that the intact YER053C and/or its degradation products have heretofore undiscovered functions in vacuoles. Nevertheless, we think that *YER053C* is most probably a rare kind of pseudogene that encodes a nonfunctional protein.

It is also noteworthy that the relationship between MIR1 and YER053C is similar to that between YSR2 andYSR3, dehydrosphingosine-1-phosphate phosphatases of *S. cerevisiae* (*45–47*). In that case, both YSR2 and YSR3 are reported to be functional *in vitro*. Intriguingly, YSR2 is localized in the endoplasmic reticulum and is essential for sphingolipid metabolism, whereas YSR3 is targeted to vacuoles and not essential for this metabolism (*47*). YSR3 may play a role in the survival of cells after heat shock (*46*).

We hypothesize that, during evolution, *S. cerevisiae* first duplicated the orthologous MPT gene. Later, one of the resulting genes, the ancestor of *YER053C*, became primarily functional, and retained typical nucleotide and amino acid sequences. The other gene, the ancestor of *MIR1*, diverged because it was not necessary for the mitochondrial function at the time. Accidentally, for an unknown reason, *S. cerevisiae* lost the function and/or normal targeting of the ancestor of the typical YER053C. Then, the yeast may have recruited the atypical *MIR1* to encode the only P_i transporter, because it is beneficial for energy metabolism. It is also noteworthy that mitochondrial ATP production is not essential for the yeast, and thus it can survive without a normal MPT function for some time. The above idea is supported by the fact that we could not find closely related homologs of MIR1 in databases, while YER053C and MPTs of other species were found with rather high scores. However, we cannot completely exclude the possibility that *YER053C* diverged very early and then accidentally came to encode an MPT-like protein of unknown function under low evolutionary pressure. Interestingly, the *YER053C* promoter still responds to aeration (Fig. 2). It has been reported that there are a number of *S. cerevisiae* genes for mitochondrial proteins, the transcription of which is regulated by the oxygen level (*48, 49*). There still remains the question of why the yeast keeps producing and then degrading YER053C of the same size with a homologous amino acid sequence as long as before.

The present-day YER053C has several unique amino acid residues compared to MPTs of various species, although they are similar to one another (Fig. 1). Also, the spacer sequence between the second and third transmembrane domains of YER053C is shorter than in other MPTs by about 6 amino acids. In the future, it may be feasible to restore the function and mitochondrial targeting of YER-053C with the aid of site-directed mutagenesis.

We thank Dr. Y. Aoyama, SokaUniversity, for the advice on anaerobic growth of yeasts.

REFERENCES

1. Pratt, R.D., Ferreira, G.C., and Pedersen, P.L. (1990) Mitochondrial phosphate transport. *J. Biol. Chem.* **266**, 1276–1280
2. Ferreira, G.C. and Pedersen, P.L. (1993) Phosphate transport in mitochondria: Past accomplishments, present problems, and future challenges. *J. Bioenerg. Biomem.* **25**, 483–492
3. Stappen, R. and Kramer, R. (1994) Kinetic mechanism of phosphate/phosphate and phosphate/OH^- antiporters catalyzed by reconstituted phophate carrier from beef heart mitochondria. *J. Biol. Chem.* **269**, 11240–11246
4. Wohlrab, H. and Briggs, C. (1994) Yeast mitochondrial phosphate transporter protein expressed in *Escherchia coli*. Site-directed mutations at threonine-43 and at a similar location in the second tandem repeat (isoleucine-141). *Biochemistry* **33**, 9371–9375
5. Schroers, A., Kramer, R., and Wohlrab, H. (1997) The reversible antiport-uniport conversion of the phosphate carrier from yeast mitochondria dependents on the presence of asingle cysteine. *J. Biol. Chem.* **272**, 10558–10564
6. Runswick, M.J., Powell, S.J., Nyren, P., and Walker, J.E. (1987) Sequence of the bovine mitochondrial phosphate carrier protein: structural relationship to ADP/ATP tranlocaseand the brown fat mitochondria uncoupling protein. *EMBO J.* **6**, 1367–1373
7. Ferreira, G.C., Pratt, R.D., and Pedersen, P.L. (1989) Energy-linked anion transport. *J. Biol. Chem.* **264**, 15628–15633
8. Dolce, V., Fiermonte, G., Messina, A., and Palmieri, F. (1991) Nucleotide sequence of a human heart cDNAencoding the mitochondrial phosphate carrier. *DNA Sequence* **2**, 133–135
9. Runswick, M.J., Philippides, A., Lauria, G., and Walker, J.E. (1994) Extension of the mitochondrial transporter super-family: Sequences of five members from the nematode worm, *Caenorhabditid elegans*. *DNA Sequence* **4**, 281–291
10. Dolce, V., Lacobazzi, V., Palmieri, F., and Walker, J.E. (1994) The sequences of human and bovinegenes of the phosphate carrier from mitochondria contain evidence of alternatively spliced forms. *J. Biol. Chem.* **269**, 10451–10460
11. Kiiskinen, M., Korhonen, M., andKangasjarvi, J. (1997) Isolation and characterization of cDNA for a plant mitochondrial phosphate translocator (*Mpt1*): Ozone stress induces *Mpt1* mRNA accumulation in birch (*Betula pendula* Roth). *Plant Mol. Biol.* **35**, 271–279
12. Takabatake, R., Hata, S., Taniguchi, M., Kouchi, H., Sugiyama, T., and Izui, K. (1999) Isolation and characterization of cDNAs encoding mitochondrial phophate transporters in soybean, maize, rice, and *Arabidopsis*. *Plant Mol. Biol.* **40**, 479–486
13. Murakami, H., Blobel, G., and Pain, D. (1990) Isolation and characterization of the gene for a yeast mitochondrial import receptor. *Nature* **347**, 488–491
14. Phelps, A., Schobert, C.T., and Wohlrab, H. (1991) Cloning and

characterization of the mitochondrial phosphate transport protein gene from yeast Saccharomyces cerevisiae. *Biochemistry* **30**, 248–252
15. Wohlrab, H., Collins, A., and Costello, D. (1984) Purified mitochondrial phosphate transport protein. improved proteoliposomes and some properties of the transport protein sulfhydryl group(s). *Biochemistry* **23**, 1057–1064
16. Ligeti, E. and Fonyo, A. (1989) Phosphate transport protein of rat heart mitochondria: location of itsSH-groups and exploration of their enviroment. *Biochim. Biophys. Acta* **973**, 170–175
17. Guerin, B., Bukusoglu, C., Rakotomanana, F., and Wohlrab, H. (1990) Mitochondrial phosphate transport. *J. Biol. Chem.* **265**, 19736–19741
18. Pain, D., Murakami, H., andBlobel, G. (1990) Identification of a receptor for protein import intomitochondria. *Nature* **347**, 444–449
19. Murakami, H., Blobel, G., and Pain, D. (1993) Signal sequence region of mitochondrial precursor proteinsbinds to mitochondrial import receptor. *Proc. Natl. Acad. Sci. USA* **90**, 3358–3362
20. Zara, V., Dietmeier, K., Palmisano, A., Vozza, A., Rassow, J., Palmieri, F., and Pfanner, N. (1996)Yeast mitochondria lacking the phosphate carrier/p32 are blocked in phosphate transport but can import preproteins after regeneration of amembrane potential. *Mol. Cell. Biol.* **16**, 6524–6531
21. Schroers, A., Burkovski, A., Wohlrab, H., and Kramer, R. (1998) The phosphate carrier from yeast mitochondria. *J. Biol. Chem.* **273**, 14269–14276
22. Hata, S., Oda, Y., Nishino, T., Katsuki, H., Aoyama, Y., Yoshida, Y., and Nagai, J. (1983) Characterization of a *Saccharomyces cerevisiae* mutant, N22, defective in ergosterol synthesis and preparation of [28-^{14}C]ergosta-5,7-dien-3β-ol with the mutant. *J. Biochem.* **94**, 501–510
23. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**, 163–168
24. Sherman, F. (1991) Getting startedwith yeast in *Methods in Enzymology* (Guthrie, C. and Fink, G.R., eds.) Vol. 194, pp. 3–21, Academic Press, New York
25. Burkholder, P.R. (1943) Vitamin deficiencies in yeasts. *Amer. J. Bot.* **30**, 206–211.
26. Elder, R.T., Loh, E.Y., and Davis, R.W. (1983) RNA from the yeast transposable element Ty*1* has both ends in the direct repeats, a structure similar to retrovirus RNA. *Proc. Natl. Acad. Sci. USA* **80**, 3358–3362
27. Philippsen, P., Stotz, A., and Scerf, C. (1991) DNA of *Saccharomyces cerevisiae* in *Methods in Enzymology* (Guthrie, C. and Fink, G.R., eds.) Vol. 194, pp. 169–182, Academic Press, New York
28. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
29. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
30. Menzlaf, E. and Flugge, U.I. (1993) Purification and functional reconstitution of the 2-oxoglutarate/malate translocator from spinach chloroplasts. *Biochim. Biophys. Acta* **1147**, 13–18
31. Preuss, D., Mulholland, J., Kaiser, C.A., Orlean, P., Albright, C., Rose, M.D., Robbins, P.W., and Botstein, D. (1991) Structure of the yeast endoplasmic reticulum:Localization of ER proteins using immunofluorescence and immunoelectron microscopy. *Yeast* **7**, 891–911
32. Ueno, O. (1992) Immunogold localization of photosynthetic enzymes in leaves of *Aristida latifolia*, a unique C4 grass with a bundle chlorenchymatous bundle sheath. *Physiol. Plant.* **85**, 189–196
33. Phelps, A., Briggs, C., Mincone,L., and Wohlrab, H. (1996) Mitochondrial phosphate transport protein. Replacements of glutamic, aspartic, and histidine residues affect transportand protein conformation and point to a coupled proton transport path. *Biochemistry* **35**, 10757–10762
34. Phelps, A. and Wohlrab, H. (1991) Mitochondrial phosphate transport. *J. Biol. Chem.* **266**, 19882–19885
35. Smith, V., Chou, K.N., Lashkari, D., Bostein, D., and Brown, P.O. (1996) Functional analysis of the genes ofyeast chromosome V by genetic footprinting. *Science* **274**, 2069–2074
36. Nelson, D.R., Felix, C.M., and Swanson, J.M. (1998) Highly conserved charge-pair networks in the mitochondrial carrier family. *J. Mol. Biol.* **277**, 285–308
37. Winzeler, E.A., Shoemaker, D.D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J.D., Bussey, H., Chu, A.M., Connelly, C., Davis, K., Dietrich, F., Dow S.W., Bakkoury, M.E., Foury, F., Friend, S.H., Gentalen, E., Giaever, G., Hagemann, J.H., Jones, T., Laub, M., Liao, H., Liebundguth, N., Lockhart, D.J., Lucau-Danila, A., Lussier, M., M'Rabet, N., Menard, P., Mittmann, M., Pai, C., Rebischung, C., Revuelta, J.L., Riles, L., Roberts, C.J., Ross-MacDonald, P., Scherens, B., Snyder, M., Sookhai-Mahadeo, S., Storms, R.K., Veronneau, S., Voet, M., Volckaert, G., Ward, T.R., Wysocki, R., Yen, G.S., Yu, K., Zimmermann, K., Philippsen, P., Johnston, M., and Davis, R.W. (1999) Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. *Science* **285**, 901–906
38. Kuan, J. and Saier, M.H., Jr. (1993) The mitochondrial carrier family of transport proteins: Structual,functional, and evolutionary relationships. *Crit. Rev. Biochem. Mol. Biol.* **28**, 209–233
39. El Moualij, B., Duyckaerts, C., Lamotte-Brasseur, J., and Sluse, F.E. (1997) Phylogenetic classification ofthe mitochondrial carrier family of *Saccharomyces cerevisiae*. Yeast 13, 573–581
40. DeRisi, J.L., Iyer, V.R., and Brown, P.O. (1997) Exploring the metabolic and genetic control of geneexpression on a genomic scale. *Science* **278**, 680–686
41. Ferea, T.L., Botstein, D., Brown, P.O., and Rosenzweig, R.F. (1999) Systematic changes in gene expressionpatterns following adaptive evolution in yeast. *Proc. Natl. Acad. Sci. USA* **96**, 9721–9726
42. ter Linde, J.J.M., Liang, H., Davis, R.W., Steensma, H.Y., van Dijken, J.P., and Pronk, J.T. (1999) Genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of *Saccharomyces cerevisiae*. *J. Bacteriol.* **181**, 7409–7413
43. Schwappach, B., Stobrawa, S., Hechenberger, M., Steinmeyer, K., and Jentsch, T.J. (1998) Golgilocalization and functionally important domains in the NH_2 and COOH terminus of the yeast CLC putative chloride channel Gef1p. *J. Biol. Chem.* **273**, 15110–15118
44. Krampe, S., Stamm, O., Hollenberg, C.P., and Boles, E. (1998) Catabolite inactivation of the high-affinityhexose transporters Hxt6 and Hxt7 of *Saccharomyces cerevisiae* occurs in the vacuole after internalization byendocytosis. *FEBS Lett.* **441**, 343–347
45. Mao, C., Wadleigh, M., Jenkins, G.M., Hannun, Y.A., and Obeid, L.M. (1997) Identification and characterization of *Saccharomyces cerevisiae* dihydrosphingosine-1-phosphate phosphatase. *J. Biol. Chem.* **272**, 28690–28694
46. Mandala, S.M., Thornton, R., Tu, Z., Kurtz, M.B., Nickels, J., Broach, J., Menzeleev, R., and Spiegel, S.(1998) Sphingoid base 1-phosphate phosphatase: A key regulator ofsphingolipid metabolism and stress response. *Proc. Natl. Acad. Sci. USA* **95**, 150–155
47. Mao, C. and Obeid, L.M. (2000) Yeast sphingosine-1-phosphate phosphatases: Assay, expression, deletion, purification, and cellular localization by GFP tagging in *Methods in Enzymology* (Merrill, A.H., Jr. and Hannun, Y.A., eds.) Vol. 311, pp. 223–233, Academic Press, New York
48. Zitomer, R.S. and Lowry, C.V. (1992) Regulation of gene expression by oxygen in *Saccharomyces cerevisiae*. *Microbiol. Rev.* **56**, 1–11
49. Gavurnikova, G., Sabova, L., Kissova, I., Haviernik, P. and Kolarov, J. (1996) Transcription of the *AAC1* gene encoding an isoform of mitochondrial ADP/ATPcarrier in *Saccharomyces cerevisiae* is regulated by oxygen in a heme-independent manner. *Eur. J. Biochem.* **239**, 759–763
50. Nei, M. (1987) *Molecular Evolutionary Genetics*, Columbia University Press, New York