ABSTRACT

Title of Dissertation: MOLECULAR ANALYSIS OF CYCLOPHILIN FUNCTION IN THE YEAST *SACCHAROMYCES CEREVISIAE*

Edward S. Davis, Doctor of Philosophy, 1994

Dissertation directed by: Miles B. Brennan, Ph.D., National Institute of Mental Health

The cyclophilins are a family of proteins first identified as receptors for cyclosporin A (CsA), a cyclic peptide of fungal origin. CsA inhibits T-lymphocyte activation, and is thus a potent immunosuppressant. Although cyclophilins are ubiquitous, and highly conserved, among eukaryotes, their normal physiological functions are unknown. As the receptors for CsA, cyclophilins might be involved in regulating signal transduction pathways. Cyclophilns also have peptidyl-prolyl, *cis-trans* isomerase (PPIase) activity *in vitro*, suggesting a role in protein folding *in vivo*. While CsA inhibits cyclophilin's PPIase activity, this inhibition is insufficient to account for the pharmacological activity of CsA. Therefore, previous results cannot be readily synthesized into a model for cyclophilin function.

The goal of this project was to define and characterize physiological roles of cyclophilins using the yeast *S. cerevisiae*. Three *S. cerevisiae* cyclophilin genes were cloned and inactivated by insertional mutagenesis. I demonstrated that one, *CPR3*, is necessary for the efficient metabolism of non-fermentable carbon sources. The *CPR3* gene product, Cpr3, is localized to the mitochondrial matrix,

and a truncated version of Cpr3 expressed in bacteria binds CsA. *CPR3* inactivation does not significantly compromise the induction of transcription of two nuclear cytochrome genes. Thus, Cpr3 is not necessary for the signal transduction pathway governing cytochrome gene expression.

To identify biochemical targets of Cpr3, I demonstrated that inactivation of a mitochondrial lactate dehydrogenase is insufficient to account for the growth defect of *cpr3* mutants. An exhaustive search for high-copy suppressors of the growth defect of *cpr3* mutants led to the identification of a novel gene, *JEN1*, that suppresses the growth defect at elevated temperature. *JEN1* encodes a protein that is probably a lactate transporter, and thus not a direct biochemical target of Cpr3. A dominant mutation in a nuclear gene, *JEN2*, suppresses the growth defect of *cpr3* mutants on lactate at 30^0C and 37^0C. *JEN2* might encode a direct biochemical target of Cpr3.

In summary, the cyclophilin, Cpr3, plays a general role in the efficient function of yeast mitochondria, and presents an excellent model system for studying cyclophilin function.

MOLECULAR ANALYSIS OF CYCLOPHILIN FUNCTION IN THE YEAST *SACCHAROMYCES CEREVISIAE*

by

Edward S. Davis

Dissertation submitted to the Faculty of the Graduate School
of The University of Maryland in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
1994

Advisory Committee:

Senior Staff Fellow Miles B. Brennan, National Institute of Mental Health, Advisor
Professor Marco Colombini, Chairman
Senior Staff Fellow Michael Lichten, National Cancer Institute
Associate Professor Spencer Benson
Associate Professor Richard Payne
Assistant Professor Soichi Tanda

U MD Dept. of Zoology

DEDICATION

This dissertation is dedicated to my loving wife, Jennifer Ann Blades. More than anyone else, Jen lived the ups and downs, the frenetic pace, and the murky uncertainty of my post-graduate odyssey. Having met and married me soon after I started this work, Jen unfairly bore the brunt of my frustrations an uncountable number of times, yet refused to give up on me. Her love sustained me through the most trying of times, especially during the writing of this document. And now, with two yeast genes named for her, she shares with me the joy of achieving my long-sought goal. Without Jen, this work would be of little significance.

ACKNOWLEDGEMENTS

Perhaps the most important lesson that I have learned over the past five years is that Science, like any other worthwhile human endeavor, cannot be done alone. From the moment I met him in New York in 1989, Miles Brennan challenged me to a standard of excellence I never thought I was capable of. At the same time, with phenomenal patience, he let me learn from many mistakes, some too embarrassing to contemplate. His unique combination of enthusiam, understanding, and leadership was essential for keeping my hopes alive at low points, and seeing me through the development of this project. I will always be profoundly grateful for this opportunity that he gave me.

The members of the laboratory, Eugene Cotton, Ute Hochgeschwender, René Hopfinger, Tim McPhail, Adelaide Robb, and Wolfgang Schwabe, were always available for discussions, advice, criticisms, emotional support, and great birthday cakes. I will miss the camaraderie of this group. Brenda Lawrence is one of my closest friends and colleagues. I could not have done without her emotional support and cheerleading.

The faculty of the Department of Zoology has provided me with invaluable guidance, advice, and support in the past three-and-one-half years, no small feat considering that I did my work on the NIH campus. I warmly thank them for accepting me as a transfer student into their eclectic program. Marco Colombini, my faculty advisor, was greatly helpful with scientific and other discussions. He was a major influence in developing my feel for an intuitive approach to scientific

problems.

I cannot be sufficiently gracious in thanking Lois Reid for all of the administrative crises she prevented me from stumbling into. Lois was always more than willing to answer any of my questions and to remind me of crucial university deadlines. Without her, I would forever be on administrative probation.

Although I am graduating from the University of Maryland, I cannot forget my first year-and-one-half in graduate school, at The Mount Sinai School of Medicine in New York City. I had many stimulating discussions with Paul Lazarow and Reza Green, which helped shape my early thinking. Jim Bieker provided valuable advice and friendship. I still appreciate the company of my old colleagues, especially Eric Brewster, John Dimari, Gene Dunkley, John Lapolla, Martin Lock, Jim Rocco, Susan Snyder, and one of my dearest friends, Michelle Dobrawsky, whose intellectual prowess I envy. And I am greatly appreciative for Ed Johnson. My years as a technician in his laboratory provided me with superb training, which aided me greatly in pursuing this work. I thank Ed, for being an early mentor, and for providing me with my first opportunity to play with test tubes for a living.

I also thank Mike Lichten of NCI, for patiently teaching me micromanipulation techniques, and for the unlimited use of his back-breaking micromanipulator. My association with Mike was instrumental in furthering my interest and facility in the yeast system.

Finally, I thank everyone in my extended family for their love and emotional support over the years. I am glad to have made them proud of me.

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

Chapter 1. Introduction

Section A: Historical Background

Cyclophilins initially attracted attention for their potential involvement in the biological actions of cyclosporin A (CsA). CsA, a hydrophobic, cyclic, undecapeptide of molecular weight 1,203 (Borel, *et al.*, 1976), is produced as a secondary metabolite by the fungi *Cylindrocarpon lucidum* and *Tolypocladium inflatum* (Borel, *et al.*, 1976). CsA was identified in fungal extracts as a fraction that, when administered to mice, inhibited antibody production and the appearance of leukocyte plaque forming cells (Borel, *et al.*, 1976).

CsA is important for clinical use because of its immunosuppressant activity. CsA is widely used to prevent rejection of organ and tissue transplants, and in treatment of autoimmune diseases and graft-versus-host reaction (Handschumacher, *et al.*, 1984).

The nature of immunosuppression is to block the activation of antigen- or mitogen-stimulated T-helper lymphocytes in response to binding at the T-cell antigen receptor (TCR). TCR-antigen binding and early T-cell signaling events are unaffected by CsA (Bijsterbosch and Klaus, 1985). The finding that CsA interacts with calmodulin (Colombani, *et al.*, 1985) suggested that CsA acts on a Ca^{2+}-dependent step in signal transduction from the TCR.

CsA is toxic to most eukaryotic cell types; in humans, therapeutic doses cause toxicity in liver, kidney, and neural tissue (Borel, *et al.*, 1977). CsA is

relatively non-toxic to muscle and myeloid tissue (Borel, *et al.*, 1977).

Section B: Discovery and distribution of cyclophilins.

Cyclophilin is a CsA-binding protein. In a search for intracellular receptors for CsA, Handschumacher, *et al.* (1984) identified two distinct 17 kd soluble proteins from bovine thymus, which they named cyclophilins, that bind to CsA; the K_d for the bovine thymus cyclophilins was measured to be 2×10^{-7} mol L^{-1} (Handschumacher, *et al.*, 1984). Subsequently, CsA binding activity was detected in a wide variety of eukaryotic species and tissues, but not in the bacterium *Escherichia coli* (Koletsky, *et al.*, 1986).

Cyclophilins are abundant, commonly comprising up to 0.1% of total cellular protein, although levels vary among cell types (Koletsky, *et al.*, 1986). The highest concentrations in humans were found in neoplastic and normal tissues of the colon, whereas the lowest were found in muscle and myeloid tissue (Koletsky, *et al.*, 1986). Sequenced cyclophilins show strong evolutionary conservation, and exist in multiple tissue- and organelle-specific isoforms. Southern blots suggest that as many as 20 to 25 cyclophilin genes exist in mammals (Haendler, *et al.*, 1987; Danielson, et. al, 1988). Further, cytosolic (Handschumacher, *et al.*, 1984), mitochondrial (Tropschug, *et al.*, 1988), endoplasmic reticulum (Hasel *et al.*, 1991), and secreted (Spik, *et al.*, 1991; Caroni, *et al.*, 1991) cyclophilin isoforms have been identified from several organisms.

Structure of cyclophilin. The three dimensional structure of unliganded, human cyclophilin A has been solved to 2.5 Å resolution, using X-ray crystallography (Ke,

et al., 1991). The structure is a compact barrel, with a hydrophobic core formed by anti-parallel ß-strands. At each end of the barrel is an α-helix. A pocket on one side harbors the CsA-binding site (Ke, *et al.*, 1991; Kallen, *et al.*, 1991).

The structure of cyclophilin bound to CsA, determined by nuclear magnetic resonance (NMR) imaging, is in good agreement with the crystal structure of unliganded cyclophilin (Thériault, *et al.*, 1993). This result indicates that there is no significant structural change in cyclophilin resulting from CsA binding. However, the structure of CsA complexed with cyclophilin differs dramatically from uncomplexed CsA (Fesik, *et al.*, 1991; Weber, *et al.*, 1991). It is unclear whether the observed structural changes in CsA are due to binding with cyclophilin, or to the fact that the complex structure was determined in aqueous solution, whereas the structure of free CsA was determined in a non-polar solvent.

Section C: Function of cyclophilins.

Despite the abundance, ubiquity, and strong evolutionary conservation of the cyclophilins, their normal physiological functions are unknown. The goal of this research has been to identify and characterize those functions.

N. crassa, selected for resistance to the cytotoxic effects of CsA, can lose immunologically detectable cyclophilin and CsA-binding activity, without loss of viability (Tropschung, *et al.*, 1989). This result suggested that cyclophilin is not essential. However, it is difficult to imagine that cyclophilin has no function. Why would an organism make a protein, comprising up to 0.1% of its total mass, that has no function? How could a gene with no function be maintained over the course of evolution? Even if investigators were to find that cyclophilins were indeed "dispensable", they might be so only under "standard laboratory conditions". However, in the laboratory one might overlook a subtle phenotype in a cyclophilin null mutant that would distinguish between survival and death in other, natural environments.

Several cyclophilin variants, reported to reside in various cellular compartments, have been identified. Therefore, it is likely that the different cyclophilin variants have functions that are important under specific physiological conditions. Thus, one particular cyclophilin might seem dispensable, unless the organism is challenged with a particular physiological stress.

1. Role of cyclophilins in the biological activities of CsA. Recently, much

progress has been made in elucidating the molecular mechanism of CsA action. In the human T-cell line Jurkat, CsA inhibits mitogen-mediated transcriptional induction of lymphokine genes, primarily interleukins 2 and 4 (IL-2 and IL-4), and interferon gamma (IFN-γ) (Krönke, *et al.*, 1984). IL-2 gene transcription requires binding of nuclear factor of activated T-cells (NFAT; Emmel, *et al.*, 1989) to the IL-2 promoter. NFAT is a multi-subunit protein, comprised of the subunits NFATp, fos, and jun (Jain, *et al.*, 1993). CsA blocks the antigen-stimulated nuclear localization of the NFATp subunit (Flanagan, *et al.*, 1991).

CsA acts in a toxic complex with cyclophilin. What roles do cyclophilins play in the biological actions of CsA? The affinity between cyclophilins and CsA initially led to speculation that CsA inhibits an essential step in signal transduction that requires a cyclophilin function. The subsequent discovery of cyclophilin's *in vitro*, CsA-sensitive, peptidyl-prolyl *cis-trans* isomerase (PPIase) activity (see below) suggested that protein folding plays an important role in signal transduction. However, some experimental evidence casts doubt that inhibition of any cyclophilin activity leads to the observed biological effects of CsA.

A synthetic analog of CsA, named MeAla-6, inhibits cyclophilin PPIase activity, but does not cause immunosuppression (Sigal, *et al.*, 1991). Further, mutants of *Neurospora crassa* selected for CsA resistance have decreased CsA binding activity, and have lost immunologically-detectable cyclophilin antigen (Tropschug, *et al.*, 1989). A similar result was obtained with a CsA-sensitive mutant of *S. cerevisiae* (Tropschug, *et al.*, 1989). These results suggest that

cyclophilin is essential for CsA action, but that CsA acts, not by inhibiting cyclophilin function, but rather through joining with cyclophilin in a complex that inhibits a component of signal transduction pathways.

Other immunosuppressants. Other immunosuppressant drugs act on signal transduction pathways. FK-506 and rapamycin are bacterial macrolide antibiotics and structurally related to one another, but not to CsA (reviewed in Sigal and Dumont, 1992). FK-506 binds to the 12-kd protein FKBP (FK-506-binding protein; Harding, *et al.*, 1989; Siekierka, et. al, 1989). FK-506, like CsA, inhibits the nuclear translocation of NFATp, and thus the assembly of NFAT, in stimulated T-lymphocytes (Flanagan, *et al.*, 1991). As with CsA, a synthetic analog of FK-506, named 506BD, inhibits PPIase activity but is nonimmunosuppressive (Bierer, *et al.*, 1990a). Thus, inhibition of T-lymphocyte activation by FK-506 is not caused by inhibition of FKBP's proline isomerase activity. Despite the functional similarity between CsA and FK-506 in blocking T-lymphocyte activation, cyclophilin and FKBP share no significant amino acid sequence similarity, and neither protein binds to the other's immunosuppressant ligand.

Rapamycin inhibits T-lymphocyte activation and binds FKBP, but, surprisingly, does not affect IL-2 gene transcription or other TCR-mediated responses (Dumont, *et al.*, 1990; Bierer, *et al.*, 1990b). Instead, rapamycin inhibits the response of T- and B-lymphocytes to IL-2, involving a signalling pathway from the IL-2 receptor (Dumont, *et al.*, 1990). Treatment of Jurkat cells with rapamycin causes a decrease in p70 s6 kinase activity, but this kinase is not a direct target of

rapamycin (Kuo, *et al.*, 1992). No direct targets of rapamycin in T-cells have yet been identified.

S. cerevisiae strains resistant to rapamycin have lost FKBP, indicating that in yeast, rapamycin may also act in a toxic complex (Heitman, *et al.*, 1991). Rapamycin sensitivity is suppressed by mutations in two putative lipid kinase genes, *TOR1* and *TOR2* (Heitman, *et al.*, 1991; Kunz, *et al.*, 1993). *TOR2*, but not *TOR1*, is essential for viability in yeast. It is not yet known if the *TOR1* and *TOR2* gene products interact directly with either FKBP or an FKBP/rapamycin complex, for it is unclear how the *tor1* and *tor2* mutations suppress rapamycin sensitivity.

Calcineurin. What cellular components do CsA/CyP complexes inhibit to mediate their biological effects? *In vitro*, a cyclophilin-glutathione-S-transferase fusion protein binds the Ca^{2+}/calmodulin-dependent, serine-threonine phosphatase calcineurin in the presence of CsA (Liu, *et al.*, 1991a). CsA inhibits calcineurin phosphatase activity *in vitro* (Liu, *et al.*, 1991a). Experiments suggest that binding of cyclophilin to calcineurin occurs through complex formation with CsA. First, binding of cyclophilin with calcineurin occurs only in the presence of CsA (Liu, *et al.*, 1991a). Second, CsA inhibits calcineurin activity only in the presence of cyclophilin (Liu, *et al.*, 1991a). An FK-506/FKBP complex also binds and inhibits calcineurin, consistent with the similar biological activities of the two drugs (Liu, *et al.*, 1991a).

In mitogen-stimulated Jurkat cells, overexpression of transfected calcineurin increases the resistance of a transfected IL-2 promoter to CsA (Clipstone and

Crabtree, 1992; O' Keefe, *et al.*, 1992). This *in vivo* result suggests that T-lymphocyte activation by the TCR is calcineurin-dependent.

It is widely suggested that a calcineurin-catalyzed dephosphorylation event is required for NFATp translocation. It is not yet known, however, if or how protein phosphorylation regulates NFAT assembly. NFATp, the NFAT subunit whose nuclear localization is CsA- and FK-506-sensitive, is a phosphoprotein that can serve as an *in vitro* substrate for calf intestinal alkaline phosphatase (McCaffrey, *et al.*, 1993a and b) (These authors present unconvincing evidence that NFATp can serve as an *in vitro* substrate for calcineurin). Further, treatment of mitogen-stimulated Jurkat cells with CsA inhibits the dephosphorylation of NFATp, suggesting that NFATp is a biochemical target of calcineurin (McCaffrey, *et al.*, 1993a).

2. PPIase activity of cyclophilins. In 1989, two groups working independently discovered that a peptidyl-prolyl *cis-trans* isomerase (PPIase, or rotamase) isolated from pig kidney is a cyclophilin (Takahashi, *et al.*, 1989; Fischer, *et al.*, 1989). PPIase activity has been observed *in vitro* to increase the rate of interconversion of prolyl amide bonds in synthetic tetrapeptides, and in some proteins, between *cis* and *trans* isomers (Fischer, *et al.*, 1989). Cyclophilin PPIase activity is inhibited by CsA; X-ray crystallographic studies indicate that the tetrapeptide model substrate for PPIase activity binds in the same area of cyclophilin as the CsA-binding site (Kallen, *et al.*, 1991). Although the rate of uncatalyzed proline isomerization under the PPIase assay conditions (aqueous solution at 10^0C) is fast

($k = 7.4 \times 10^{-3}\ s^{-1}$), PPIase causes a 135-fold increase in the isomerization rate (Fischer, *et al.*, 1989).

PPIase activity has subsequently been observed in all cyclophilin proteins that have been tested. Two proteins with PPIase activity have been identified in *E. coli* (Kawamukai, *et al.*, 1989; Hayano, *et al.*, 1991). These proteins have weak (*ca.* 30%) amino acid identity with cyclophilins. The PPIase activity of the periplasmic form is *ca.* 500-fold less sensitive to CsA than that of human cyclophilin A (Liu, *et al.*, 1991b). Mutation of a phenylalanine to tryptophan at position 112 of one of the *E. coli* proteins causes a 23-fold increase in the CsA-sensitivity of its PPIase activity (Liu, et. al, 1991b). Conversely, mutation of the highly conserved tryptophan residue at position 121 in human cyclophilin A to phenylalanine results in a 75-fold reduction in its CsA-sensitivity, but only a 2-fold decrease in PPIase activity, indicating that the CsA-binding and PPIase activities are not entirely coincident physically, nor physically separated (Liu, et. al, 1991b).

Role of cyclophilins in protein folding. A simple model for cyclophilin function suggests a role for catalyzing protein folding *in vivo*. One argument against this model is that proline isomerization under physiological conditions may occur too quickly for catalysis to be relevant to protein folding. However, the standard PPIase assay is performed using a tetrapeptide in free solution as substrate (Fischer, *et al.*, 1989). While the spontaneous proline isomerization rate under these conditions might be fast, a proline in a globular protein might be surrounded by other domains that would place physical or steric constraints on it. These

constraints would greatly reduce the spontaneous proline isomerization rate, perhaps so much that catalysis would be required for efficient folding under physiological conditions. Indeed, in the *in vitro* refolding of some denatured globular proteins (e. g. bovine carbonic anhydrase B), proline isomerization occurs as a slow ($t_{1/2}$= 120-600 sec.), late-stage, rate-limiting step (Semisotnov, *et al.*, 1990).

Further, only a small number of denatured proteins refold spontaneously *in vitro*, *e. g.* ribonuclease A (Anfinsen, 1973), bovine carbonic anhydrase B (Semisotnov, *et al.*, 1990), bovine α-lactalbumin (Ikeguchi, *et al.*, 1986), and bovine rhodanese (Mendoza, *et al.*, 1991). However, proteins may fold quite differently *in vivo* than *in vitro*; *in vitro* protein folding often requires non-physiological conditions. For example, denatured bovine rhodanese will refold spontaneously at 10^0C, but not at 37^0C (Mendoza, *et al.*, 1991).

Finally, some denatured proteins refold spontaneously *in vitro*, but not *in vivo*. Mouse dihydrofolate reductase (DHFR) will refold spontaneously after denaturation (Eilers *et al.*, 1988). If DHFR is imported into *N. crassa* mitochondria, however, its folding requires ATP hydrolysis (Ostermann, *et al.*, 1989). Other proteins imported into yeast mitochondria require hsp60 and hsp70 (known as "molecular chaperones"; see below) for proper folding (Cheng, *et al.*, 1989; Kang, *et al.*, 1990). Clearly, the efficient folding of many proteins *in vivo* is not spontaneous, but requires additional cellular factors.

Molecular chaperones. Much evidence for assisted protein folding *in vivo* comes

from work on the so-called "molecular chaperones" (reviewed in Gething and Sambrook, 1992). These diverse proteins comprise three large families, based on molecular weight; i. e., the 90, 70, and 60 kd classes. Proteins from each of these classes show strong evolutionary conservation, having been found in both prokaryotes and eukaryotes. Further, like the cyclophilins, eukaryotic molecular chaperones have been identified in numerous organelles and subcellular compartments.

Some of the molecular chaperones were formerly known as "heat shock proteins", because their synthesis increased after exposure of cells to higher than normal temperatures. These results suggested that molecular chaperones function to bind unfolded or misfolded proteins, in order to prevent their aggregation and degradation. However, more recent work demonstrated that these proteins serve more functions than response to conditions of cellular stress; for example, in *S. cerevisiae*, the endoplasmic reticulum protein Kar2 (the *S. cerevisiae* homolog of immunoglobulin heavy chain binding protein, or BiP) and the mitochondrial proteins Ssc1 (mhsp70) and mhsp60 (chaperonin 60) are constitutively expressed and essential for viability (Craig, *et al.*, 1989; Reading, *et al.*, 1989; Nicholson, *et al.*, 1990).

The physiological functions of the molecular chaperones are not well understood in biochemical detail. Some results from yeast suggest that the cytosolic molecular chaperones have "antifolding" activity, that is, newly synthesized proteins are kept unfolded to prevent misfolding or aggregation during protein synthesis or transport to another part of the cell (Gething and Sambrook,

1992).

Even more progress concerning the functions of molecular chaperones comes from work in bacteria and in mitochondria of *S. cerevisiae* and *N. crassa*. Temperature-sensitive yeast mutants were used to show that Ssc1 and mhsp60, both mitochondrial matrix proteins, are required for the import into and proper folding of proteins in mitochondria (Kang, *et al.*, 1990). In *S. cerevisiae mif4* (*hsp60*) temperature-sensitive mutants, proteins could be imported, at the non-permissive temperature, into mitochondria and processed to mature size; proper folding, however, did not occur (Cheng, *et al.*, 1989). These results suggest that Ssc1 and mhsp60 function in protein folding in mitochondria. mhsp60-mediated protein folding requires ATP hydrolysis (Ostermann, *et al.*, 1989), suggesting that mhsp60 actually catalyzes protein folding. Alternatively, mhsp60 might act as a "scaffold" upon which protein folding occurs.

How do mhsp60 and mhsp70 act in mitochondria to facilitate protein folding? A few clues come from work on *in vitro* reconstitution studies using bacterial proteins. Some *E. coli* molecular chaperones are highly homologous to eukaryotic mitochondrial proteins. For example, the *E. coli* protein DnaK shares 58% amino acid identity with *S. cerevisiae* Ssc1 (Craig, *et al.*, 1989), and the *E. coli* GroEL shares 54% amino acid identity with *S. cerevisiae* mhsp60 (Reading, *et al.*, 1989).

Proteins unfolded by urea bind to the bacterial mhsp70 equivalent, DnaK, *in vitro*, but do not refold until after the sequential addition of GrpE, GroEL, GroES, and ATP (Langer, *et al.*, 1992). Addition of unfolded proteins to any of

these components in a different sequence does not yield properly folded protein. These results gave rise to the following model for bacterial protein processing: Newly synthesized proteins are bound, and kept, in extended conformation by the "antifolding" activity of the DnaK/DnaJ complex. GrpE, by an unknown mechanism, transfers the polypeptide to GroEL. In the presence of GroEL-catalyzed ATP hydrolysis, stabilized by GroES, folding is completed, and the mature protein is released (Langer, *et al.*, 1992).

Because of the high degree of similarity between the prokaryotic and eukaryotic molecular chaperones, a mechanism similar to that described above may occur during import of proteins into mitochondria. Additional work is required to further define mechanisms of mitochondrial protein folding.

Role of proline isomerization in protein folding. Where does proline isomerization fit into a general model of protein folding? Currently, the most widely accepted view of *in vitro* protein folding mechanisms is the "framework model" (reviewed in Kim and Baldwin, 1990). In the framework model, protein folding occurs as a two- or multi-stage process: A fully denatured protein, when allowed to refold, first forms regions of secondary structure (*e. g.* α-helix, ß-sheet), well before native-like tertiary interactions (interactions between secondary structures) occur. The initial secondary structure formation is fast, and not rate-limiting; it behaves essentially as a first-order reaction. The completed domains then collide or interact with one another to form the final product.

In the *in vitro* unfolding and refolding of some proteins, a transient, native-

like intermediate, termed "molten globule", forms (Wong and Tanford, 1973; Kuwajima, *et al.*, 1976; Ptitsyn, *et al.*, 1990). As in the framework model, native-like secondary structure formation occurs first, followed by the intermediate. Spectroscopic results demonstrate that although the molten globule contains native-like secondary structure, it does not have native-like tertiary structure. The intermediate is compact (just slightly larger than native), due to a collapse of hydrophobic regions away from the solvent. Formation of the molten globule occurs extremely quickly, usually within 20 msec (Ptitsyn, *et al.*, 1990).

Once the intermediate has formed, the various domains are in a transient, fluctuating state, until formation of the native state is complete. While the ΔG of folded proteins is lower than that of unfolded, random-coils, the initial stages of protein refolding are so fast that they are probably driven kinetically (Kim and Baldwin, 1990); a protein could not possibly form all of the possible thermodynamically stable structures in a pathway leading to the native state. The later stages of protein refolding are much slower, however. It is in these latter stages where certain types of modifications, *e. g.* disulfide bond formation and proline isomerization, may be rate-limiting (Lang and Schmid, 1988).

ninaA. Some evidence supporting an *in vivo* protein folding mechanism for cyclophilins comes from the fruit fly, *D. melanogaster*. Shieh, *et al.* (1989) found that the *ninaA* gene, which is essential for proper visual transduction, encodes a cyclophilin-related (44% amino acid identity to bovine cyclophilin) protein. The *ninaA* gene product is a photoreceptor cell-specific, integral membrane cyclophilin

(Stamnes, *et al.*, 1991).

NinaA mutants have normal levels of opsin mRNAs, which encode rhodopsins in the R1-R6 photoreceptor cells, but are deficient in Rh1 rhodopsin protein levels as determined by Western blotting (Shieh, *et al.*, 1989). *NinaA* acts specifically on Rh1 rhodopsin; levels of the other rhodopsins, Rh2, Rh3, and Rh4, are unaffected in *ninaA* mutants (Stamnes, et. al, 1991). Finally, *ninaA* is required for transport of Rh1 through the secretory pathway (Colley, *et al.*, 1991). These results suggest that this cyclophilin plays a post-transcriptional, and perhaps post-translational, role in the maturation of a particular protein. However, it is not yet known if the *ninaA* gene product interacts directly with Rh1 rhodopsin.

Section D: Determining cyclophilin function.

I have been interested in determining and characterizing the functions of the cyclophilins. In approaching this question, the work cited in this chapter presents some problems. First, the requirement for cyclophilin in the biological actions of CsA suggests that they might play essential roles in signal transduction pathways. Some attention has been devoted to a model whereby cyclophilin might naturally regulate calcineurin activity, via endogenous ligands similar to CsA, as has been recently suggested (Steiner, *et al.*, 1992). Thus, cyclophilins could play a natural inhibitory role in the cell under specific physiological conditions.

Secondly, the *in vitro* proline isomerase activity of cyclophilins, and the results of the *D. melanogaster ninaA* mutant, suggest that proline isomerization and protein folding might play a role in cyclophilin function. However, since CsA-resistant mutants of *N. crassa* have lost cyclophilin, and since synthetic analogs of CsA and FK-506 that inhibit PPIase are no longer immunosuppressive (Bierer, *et al.*, 1990a; Sigal, *et al.*, 1991), inhibition of cyclophilin PPIase activity cannot account for the observed biological actions of CsA (Tropschug, *et al.*, 1989).

Thus, it is difficult to resolve these findings as they relate to cyclophilin function, for they appear contradictory. In one scenario, cyclophilins have been observed to play a part in signal transduction pathways. However, in this case, they are mediating the biological actions of a compound (CsA) that is not a normal physiological component of eukaryotic cells. Further, the biological actions of CsA and FK-506 are very similar: Inhibition of calcineurin-mediated

signal transduction. The primary structures of cyclophilin and FK-506 have no apparent similarity, but the three-dimensional structures of the cyclophilin/CsA complex is remarkably similar to the FKBP/FK-506 complex (Schreiber, 1992).

Finally, rapamycin, another substance not normally found in eukaryotic cells, binds FKBP, but inhibits an unknown signal transduction pathway distinct from that inhibited by FK-506 (Bierer, *et al.*, 1990b). Thus, the biological similarities between CsA and FK-506 suggests that binding of immunosuppressant drugs by immunophilins may be a form of molecular mimicry: Cyclophilins and FKBPs have been conserved for functions other than immunosuppressant drug actions, but these drugs use "immunophilins" (the immunosuppressant-binding proteins cyclophilin and FKBP) as a way to exert their effects. An analogy can be drawn from virology, where the cellular receptors for virus are maintained for a function other than facilitating virus entry. Therefore, binding of CsA to cyclophilin action may simply be a means to an end by which microorganisms co-opt an existing structure, and may not be relevant to the normal physiological functions of cyclophilins.

This model can be illustrated by the results from mutations in human cyclophilin A, outlined in Section C: A mutation in the highly conserved tryptophan at position 121 to phenylalanine causes a 75-fold reduction in CsA binding activity, but only a two-fold reduction in PPIase activity (Liu, *et al.*, 1991b). Because this tryptophan is highly conserved, however, it may have been selected for a function other than PPIase. Thus, the structure of the CsA binding site has been conserved as well, and is exploited by these immunosuppressant compounds.

Alternatively, PPIase activity may be artifactual; it has only been observed *in vitro*. The Rh1 rhodopsin deficiency of *Drosophilia ninaA* mutants is the only case supportive of an *in vivo* protein folding role for cyclophilins. Yet, even in this case, a physical interaction between the *ninaA* gene product and Rh1 rhodopsin has not been demonstrated, and it is not yet known if the *ninaA* protein has PPIase activity. So, the physiological relevance of PPIase activity is open to question as well.

I decided that the best way to address the goals of this project was to determine the natural physiological roles of cyclophilins. This would require identifying both physiological conditions for which cyclophilins are essential, and biochemical factors cyclophilins interact with.

I decided to pursue a genetic approach to the problem of determining cyclophilin function; i. e., to construct cyclophilin mutants, identify phenotypes resulting from these mutations, and study these phenotypes. Ultimately, when phenotypes are characterized, they can be suppressed by mutations at second-sites (rescue of the phenotype mediated by a gene other than the cyclophilin gene being studied). The aim of this approach is to determine the function of cyclophilins, and to identify either cyclophilin substrates, or natural ligands that regulate cyclophilin activity.

The *ninaA* gene product is the only cyclophilin with a known physiological function. While the *D. melanogaster* system is a well-characterized experimental system, it is still not facile to studying physiology or biochemistry at the cellular level. Similarly, it would be impractical to use a genetic approach to the problem

of determining cyclophilin function in a mammalian system, due to the genetic complexity of mammalian cyclophilins.

I chose the yeast, *S. cerevisiae*, as an experimental system for determining cyclophilin function, as CsA-binding activity had been reported in extracts from yeast cells (Koletsky, et. al, 1986). *S. cerevisiae* is a single-celled fungus with a short life cycle (generation times range from 1.3 to 10 hours, depending on growth conditions). This rapid generation time makes *S. cerevisiae* an ideal organism for biochemical experiments. *S. cerevisiae* has a small genome size (1.4×10^7 bp, or 100-fold smaller than human) and a well-characterized genetic system (Roman, *et al.*, 1981). Thus, all classical as well as molecular genetic manipulations can be performed readily in this organism.

It will be useful to determine the functions of cyclophilins, as this could help in studying how cyclophilins mediate the biological actions of CsA. Additionally, research into cyclophilin function could be beneficial to other areas of research, particularly protein folding and organelle biogenesis.

References

Anfinsen, C. B. (1973). Principles that govern the folding of protein chains. *Science* **181**, 223-230.

Bierer, B. E., Somers, P. K., Wandless, T. J., Burakoff, S. J., & Schreiber, S. L. (1990a). Probing immunosuppressant action with a nonnatural immunophilin ligand. *Science* **250**, 556-559.

Bierer, B. E., Mattila, P. S., Standaert, R. F., Herzenberg, L. A., Burakoff, S. J., Crabtree, G. R., & Schreiber, S. L. (1990b). Two distinct signal transduction pathways in T-lymphocytes are inhibited by complexes formed between an immunophilin and either FK-506 or rapamycin. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 9231-9235.

Bijsterbosch, M. K. and Klaus, G. G. (1985). Cyclosporine does not inhibit mitogen-induced inositol phospholipid degradation in mouse lymphocytes. *Immunology* **56**, 435-440.

Borel, J. F., Feurer, C., Gubler, H. U., & Staehlein, H. (1976). Biological effects of cyclosporin A: A new anti-lymphocytic agent. *Agents and Actions* **6**, 468-475.

Borel, J. F., Feurer, C., Magnee, C. & Staehlein, H. (1977). Effects of the new

anti-lymphocytic agent peptide cyclosporin A in animals. *Immunology* **32**, 1017-1025.

Caroni, P., Rothenfluh, A., McGlynn, E., & Schneider, C. (1991). S-cyclophilin. New member of the cyclophilin family associated with the secretory pathway. *J. Biol. Chem.* **266**, 10739-10742.

Cheng, M. Y., Hartl, F.-U., Martin, J., Pollock, R. A., Kalousek, F., Neupert, W., Hallberg, E. M., Hallberg, R. L., & Horwich, A. L. (1989). Mitochondrial heat-shock protein hsp60 is essential for the assembly of protiens imported into yeast mitochondria. *Nature* **337**, 620-625.

Clipstone, N. A. and Crabtree, G. R. (1992). Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* **357**, 695-697.

Colley, N. J., Baker, E. K., Stamnes, M. A., & Zuker, C. S. (1991). The cyclophilin homolog *ninaA* is required in the secretory pathway. *Cell* **67**, 255-263.

Colombani, P. M., Robb, A., & Hess, A. D. (1985). Cyclosporin A binding to calmodulin: A possible site of action on T lymphocytes. *Science* **228**, 337-339.

Craig, E. A., Kramer, J., Shilling, J., Werner-Washburne, M., Holmes, S., Kosic-Smithers, J., & Nicolet, C. M. (1989). *SSC1*, an essential member of the yeast

hsp70 multigene family, encodes a mitochondrial protein. *Mol. Cell. Biol.* **9**, 3000-3008.

Danielson, P. E., Forss-Petter, S., Brow, M. A., Calavetta, L., Douglass, J., Milner, R. J., & Sutcliffe, J. G. (1988). p1B15: A cDNA clone of the rat MRNA encoding cyclophilin. *DNA* **7**, 261-267.

Dumont, F. J., Staruch, M. J., Koprak, S. L., Melino, M. R., & Sigal, N. H. (1990). Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin. *J. Immunol.* **144**, 251-258.

Eilers, M., Hwang, S., & Schatz, G. (1988). Unfolding and refolding of a purified precursor protein during import into isolated mitochondria. *EMBO J.* **7**, 1139-1145.

Emmel, E. A., Verweij, C. L., Durand, D. B., Higgins, K. M., Lacy, E., & Crabtree, G. R. (1989). Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. *Science* **246**, 1617-1620.

Fesik, S. W., Gampe, Jr., R. T., Eaton, H. L., Gemmecker, G., Olejniczak, E. T., Neri, P., Holzman, T. F., Egan, D. A., Edalji, R., Simmer, R., Helfrich, R., Hochlowski, J., & Jackson, M. (1991). NMR studies of [U-^{13}C]cyclosporin A bound to cyclophilin: Bound conformation and portions of cyclosporin involved

in binding. *Biochemistry* **30**, 6574-6583.

Fischer, G., Wittman-Liebold, B., Lang, K., Kiefhaber, T., & Schmid, F. X. (1989). cyclophilin and peptidyl-prolyl *cis-trans* isomerase are probably identical proteins. *Nature* **337**, 476-478.

Flanagan, W. M., Corthesy, B., Bram, R. J., & Crabtree, G. R. (1991). Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature* **352**, 803-807.

Gething, M.-J. & Sambrook, J. (1992). Protein folding in the cell. *Nature* **355**, 33-45.

Haendler, B., Hofer-Warbinek, R., & Hofer, E. (1987). Complementary DNA for human T-cell cyclophilin. *EMBO J.* **6**, 947-950.

Handschumacher, R. E., Harding, M. W., Rice, J., Drugge, R. J., & Speicher, D. W. (1984). Cyclophilin: A specific cytosolic binding protein for cyclosporin A. *Science* **226**, 544-547.

Harding, M. W., Galat, A., Uehling, D. E., & Schreiber, S. L. (1989). A receptor for the immunosuppressant FK506 is a *cis-trans* peptidyl-prolyl isomerase. *Nature* **341**, 758-760.

Hasel, K. W., Glass, J. R., Godbout, M., & Sutcliffe, J. G. (1991). An endoplasmic reticulum-specific cyclophilin. *Mol. Cell. Biol.* **11**, 3484-3491.

Hayano, T., Takahashi, N., Kato, S., Maki, N., & Suzuki, M. (1991). Tow distinct forms of peptidylprolyl-*cis-trans* isomerase are expressed separately in periplasmic and cytoplasmic compartments of *Escherichia coli* cells. *Biochemistry* **30**, 3041-3048.

Heitman, J., Movva, N. R., & Hall, M. N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* **253**, 905-909.

Ikeguchi, M., Kuwajima, K., Mitani, M., & Sugai, S. (1986). Evidence for identity between the equilibrium unfolding intermediate and a transient folding intermediate: A comparative study of the folding reactions of α-lactalbumin and lysozyme. *Biochemistry* **25**, 6965-6972.

Jain, J., McCaffrey, P. G., Valge-Archer, V. E., & Rao, A. (1993). Nuclear factor of activated T cells contains fos and jun. *Nature* **356**, 801-804.

Kim, P. S. & Baldwin, R. L. (1990). Intermediates in the folding of small proteins. *Ann. Rev. Biochem.* **59**, 631-660.

Kallen, J., Spitzfaden, C., Zurini, M. G. M., Wider, G., Widmer, H., Wüthrich, K.,

& Walkinshaw, M. D. (1991). Structure of human cyclophilin and its binding site for cyclosporin A determined by X-ray crystallography and NMR spectroscopy. *Nature* **353**, 276-279.

Kang, P.-J., Ostermann, J., Shilling, J., Neupert, W., Craig, E. A., & Pfanner, N. (1990). Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. *Nature* **348**, 137-143.

Kawamukai, M., Matsuda, H., Fujii, W., Utsumi, R., & Komano, T. (1989). Nucleotide sequences of *fic* and *fic-1* genes involved in cell filamentation induced by cyclic AMP in *Escherichia coli*. *J. Bacteriol.* **171**, 4525-4529.

Ke, H., Zydowsky, L. D., Liu, J., & Walsh, C. T. (1991). Crystal structure of recombinant human T-cell cyclophilin at 2.5 Å resolution. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 9483-9487.

Koletsky, A. J., Harding, M. W., & Handschumacher, R. E. (1986). Cyclophilin: Distribution and variant properties in normal and neoplastic tissues. *J. Immunol.* **137**, 1054-1059.

Krönke, M. Leonard, W. J., Depper, J. M., Arya, S. K., Wong-Staal, F., Gallo, R. C., Waldmann, T. A., & Greene, W. C. (1984). Cyclosporin A inhibits T-cell growth factor gene expression at the level of MRNA transcription. *Proc. Natl.*

Acad. Sci. U. S. A. **81**, 5214-5218.

Kunz, J., Henriquez, R., Schneider, U., Deuter-Reinhard, M., Movva, N. R., & Hall, M. N. (1993). Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G_1 progression. *Cell* **73**, 585-596.

Kuwajima, K., Nitta, K., Yoneyama, M., & Sugai, S. (1976). Three-state denaturation of α-lactalbumin by guanidine hydrochloride. *J. Mol. Biol.* **106**, 359-373.

Kuo, C. J., Chung, J., Fiorentino, D. F., Flanagan, W. M., Blenis, J., & Crabtree, G. R. (1992). Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. *Nature* **358**, 70-73.

Lang, K. & Schmid, F. X. (1988). Protein-disulfide isomerase and prolyl isomerase act differently and independently as catalysts of protein folding. *Nature* **331**, 453-455.

Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K., & Hartl, F-U. (1992). Successive action of DnaK, DnaJ, and GroEL along the pathway of chaperone-mediated protein folding. *Nature* **356**, 683-689.

Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I., & Schreiber,

S. L. (1991a). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**, 807-815.

Liu, J., Chen, C-M., & Walsh, C. T. (1991b): Human and *Escherichia coli* cyclophilins: Sensitivity to inhibition by the immunosuppressant cyclosporin A correlates with a specific tryptophan residue. *Biochemistry* **30**, 2306-2310.

Lubben, T. H., Gatenby, A. A., Donaldson, G. K., Lorimer, G. H., & Viitanen, P. V. (1990). Identification of a groES-like chaperonin in mitochondria that facilitates protein folding. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 7683-7687.

McCaffrey, P. G., Perrino, B. A., Soderling, T. R., & Rao, A. (1993a). NFATp, a T lymphocyte DNA-binding protein that is a target for calcineurin and immunosuppressive drugs. *J. Biol. Chem.* **268**, 3747-3752.

McCaffrey, P. G., Luo, C., Kerppola, T. K., Jain. J., Badalian, T. M., Ho, A. M., Burgeon, E., Lane, W. S., Lambert, J. N., Curran, T., Verdine, G. L., Rao, A., & Hogan, P. G. (1993b). Isolation of the Cyclosporin-sensitive T cell transcription factor NFATp. *Science* **262**, 750-754.

Mendoza, J. A., Rogers, E., Lorimer, G. H., & Horowitz, P. M. (1991). Unassisted refolding of urea unfolded rhodanese. *J. Biol. Chem.* **266**, 13587-13591.

Nicholson, R. C., Williams, D. B., & Moran, L. A. (1990). An essential member of the HSP70 gene family of *Saccharomyces cerevisiae* is homologous to immunoglobulin heavy chain binding protein. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 1159-1163.

O′ Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J., & O′Neill, E. A. (1992). FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. *Nature* **357**, 692-694.

Ostermann, J. Horwich, A. L., Neupert, W., & Hartl, F.-U. (1989). Protein folding in mitochondria requires complex formation with hsp60 and ATP hydrolysis. *Nature* **341**, 125-130.

Ptitsyn, O. B., Pain, R. H., Semisotnov, G. V., Zerovnik, E., & Razgulyaev, O. I. (1990). Evidence for a molten globule state as a general intermediate in protein folding. *FEBS Lett.* **262**, 20-24.

Reading, D. S., Hallberg, R. L., & Myers, A. M. (1989). Characterization of the yeast *HSP60* gene encoding for a mitochondrial assembly factor. *Nature* **337**, 655-659.

Roman, H. (1981). Development of yeast as an experimental organism. In The Molecular Biology of the yeast Saccharomyces, volume I: Life cycle and

inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schreiber, S. L. (1992). Immunophilin-sensitive protein phosphatase action in cell signaling pathways. *Cell* **70**, 365-368.

Semisotnov, G. V., Uversky, V. N., Sokolovsky, I. V., Gutin, A. M., Razgulyaev, O. I., & Rodionova, N. A. (1990). Two slow stages in refolding of bovine carbonic anhydrase B are due to proline isomerization. *J. Mol. Biol.* **213**, 561-568.

Shieh, B-H., Stamnes, M. A., Seavello, S., Harris, G. L., & Zuker, C. S. (1989). The *ninaA* gene required for visual transduction in *Drosophila* encodes a homolog of cyclosporin A-binding protein. *Nature* **338**, 67-70.

Siekierka, J. J., Hung, S. H. Y., Poe, M., Lin, C. S., & Sigal, N. H. (1989). A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl *cis-trans* isomerase activity but is distinct from cyclophilin. *Nature* **341**, 755-757.

Sigal, N. H., Dumont, F., Durette, P., Siekierka, J. J., Peterson, L., Rich, D. H., Dunlap, B. E., Staruch, M. J., Melino, M. R., Koprak, S. L., Williams, D., Witzel, B., & Pisano, J. M. (1991). Is cyclophilin involved in the immunosuppressive and nephrotoxic action of cyclosporin A? *J. Exp. Med.* **173**, 619-628.

Sigal, N. H. & Dumont, F. J. (1992). Cyclosporin A, FK-506, and rapamycin:

Pharmacologic probes of lymphocyte signal transduction. *Ann. Rev. Immunol.* **10**, 519-560.

Spik, G., Haendler, B., Delmas, O., Mariller, C., Chamoux, M., Maes, P., Tartar, A., Montreuil, J., Stedman, K., Kocher, H. P., Keller, R., Hiestand, P. C., & Movva, N. R. (1991). A novel secreted cyclophilin-like protein (SCYLP). *J. Biol. Chem.* **266**, 10735-10738.

Stamnes, M. A., Shieh, B-H., Chuman, L., Harris, G. L., & Zuker, C. S. (1991). The cyclophilin homolog *ninaA* is a tissue-specific integral membrane protein required for the proper synthesis of a subset of *Drosophila* rhodopsins. *Cell* **65**, 219-227.

Steiner, J. P., Dawson, T. M., Fotuhi, M., Glatt, C. E., Snowman, A. M., Cohen, N., & Snyder, S. H. (1992). High brain densities of the immunophilin FKBP colocalized with calcineurin. *Nature* **358**, 584-587.

Takahashi, N., Hayano, T., & Suzuki, M. (1989). Peptidyl-prolyl *cis-trans* isomerase is the cyclosporin A-binding protein cyclophilin. *Nature* **337**, 473-475.

Thériault, Y., Logan, T. M., Meadows, R., Yu, L., Olejniczak, E. T., Holzman, T. F., Simmer, R. L., & Fesik, S. (1993). Solution structure of the cyclosporin A/cyclophilin complex by NMR. *Nature* **361**, 88-91.

Tropschug, M., Nicholson, D. W., Hartl, F-U., Köhler, H., Pfanner, N., Wachter, E., & Neupert, W. (1988). Cyclosporin A-binding protein (cyclophilin) of *Neurospora crassa*. *J. Biol. Chem.* **263**, 14433-14440.

Tropschug, M., Barthelmess, I. B., & Neupert, W. (1989). Sensitivity to cyclosporin A is mediated by cyclophilin in *Neurospora crassa* and *Saccharomyces cerevisiae*. *Nature* **342**, 953-955.

Weber, C., Wider, G., von Freyberg, B., Traber, R., Braun, W., Widmer, H., & Wüthrich, K. (1991). The NMR structure of cyclosporin A bound to cyclophilin in aqueous solution. *Biochemistry* **30**, 6563-6574.

Wong, K.-P. & Tanford, C. (1973). Denaturation of bovine carbonic anhydrase B by guanidine hydrochloride. *J. Biol. Chem.* **248**, 8518-8523.

Chapter 2. Isolation of Cyclophilin Genes From Yeast: A Yeast Cyclophilin Gene Essential For Lactate Metabolism At High Temperature

Summary

The cyclophilins are a family of ubiquitous eukaryotic proteins first identified by high affinity for cyclosporin A (CsA). The immunosuppressant and cytotoxic effects of CsA are thought to result from formation of a toxic complex between cyclophilin and CsA rather than from inhibition of cyclophilin function. The physiological role(s) of the cyclophilins are unknown. Cyclophilins have *in vitro* peptidyl-prolyl cis-trans isomerase (PPIase) activity, and thus may be involved in protein folding *in vivo*. We have isolated a novel yeast cyclophilin gene, *CPR3*, which encodes a presumptive mitochondrial isoform. While *CPR3* disruption mutants lack any phenotype at 30^0C, they are unable to grow on L-lactate at 37^0C. Disruptions of two other cyclophilin genes (*CPR1*, *CPR2*) and of *FPR1*, the gene encoding an FK506 binding protein with PPIase activity, do not affect growth on L-lactate at 37^0C. L-lactate metabolism requires the transcriptional induction of *CYB2*, the gene encoding flavocytochrome b_2; cpr3 mutants induce transcription of this gene normally. This result demonstrates a conditional lethal phenotype for a cyclophilin mutation and presents a system for genetic and biochemical analysis of cyclophilin function.

Introduction

Cyclosporin A (CsA), a secondary metabolite of *Tolypocladium inflatum*, is a hydrophobic, undecameric, cyclic peptide with both immunosuppressant and cytotoxic activities (1). Proteins with high affinity for CsA, termed cyclophilins, have been purified from a number of eukaryotic sources (2-8). While cyclophilins from distantly related species have high sequence identity (2-8), multiple isoforms, including cytosolic (2, 3, 6, 7), mitochondrial (6), secretory pathway (4, 5, 9), and secreted forms (10, 11), have been found. In addition, a cyclophilin homolog which does not bind CsA has been identified in *Escherichia coli* (12). Two lines of evidence suggest that cyclophilins are the receptors for CsA. First, *Neurospora crassa* mutants selected for CsA resistance have lost CsA binding activity and, in some cases, also lack immunologically detectable cyclophilin (13). Second, *in vitro*, a complex of cyclophilin and CsA binds specifically to calcineurin, a calmodulin-dependent serine-threonine phosphatase (15). These results suggest that the complex formed between cyclophilin and CsA is toxic through interaction(s) with a third component, rather than by inhibition of a cyclophilin function.

The physiological roles of cyclophilins, as opposed to their role in mediating CsA cytotoxicity, are unknown. *In vitro*, cyclophilins have peptidyl prolyl cis-trans isomerase (PPIase), or rotamase, activity (16, 17). Although the rate of proline isomerization is quite fast under normal physiological conditions (18), cyclophilins may be involved in the folding of proteins associated with chaperonins. One finding suggestive of such a role is the *ninaA* mutation of

Drosophila melanogaster. The *ninaA* gene encodes a rhabdomere-specific cyclophilin necessary for the proper post-translational processing of rhodopsin (4, 5).

We report that a yeast cyclophilin gene, *CPR3*, is essential for growth on L-lactate at 37^0C, and we demonstrate a conditional lethal phenotype for a cyclophilin mutation.

Materials and methods

Yeast Strains and Genetic Manipulations. Strain CMY214 (*MAT***a**/*MATα ade2-101/ade2-101 trp1-Δ1/trp1-Δ1 ura3-52/ura3-52 his3-Δ200/his3-Δ200 lys2-801/lys2-801 CAN1/can1*) was kindly provided by Dr. Carl Mann (Centre d'Etudes de Saclay). Haploid strain MB11 (Table 1) was derived from CMY214. The quadruple disruption series was derived from strain MH272-3c (*MAT***a** *trp1-1 his3 leu2-3 112 ura3-52 HML***a**). The *cpr1::LEU2* and *fpr1::URA3* alleles have been described previously (19). Yeast genetic manipulations were performed as described (20). Rho$^-$ yeast strains were selected following ethidium bromide treatment as described (20). CsA binding assays were performed as described (2).

Library construction and screening. The protocols for genomic library construction have been published previously (21). DNA from *Saccharomyces cerevisiae* strain S288C was partially digested with the restriction enzyme Sau3AI, and the 9- to 23 kilobase fraction was gel purified and ligated into phage vector Lambda DASH (Stratagene). The ligated DNA was packaged *in vitro* (Gigapack; Stratagene) and plated on *E. coli* strain LE392/P2. Plaques were transferred to nitrocellulose and hybridized at 60^{0}C with 1B15, a rat cyclophilin cDNA clone (22) labeled with ^{32}P by the random hexamer method (23). The hybridization mixture was 5X SSC (1X SSC is 0.15M sodium chloride, 0.015M sodium citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS), 5X Denhardt's solution (5X Denhardt's solution is 0.1% each Ficoll 400, polyvinylpyrrolidone, and bovine serum albumin),

and 100 μg ml^{-1} sonicated, denatured salmon sperm DNA. After hybridization, the filters were washed in 2X SSC, 0.1% SDS at 55^0C. Two different sets of overlapping clones were identified by restriction analysis.

DNA Sequencing. A 622 bp AccI fragment containing the *CPR3* gene was cloned in both orientations into M13mp18. Sequencing was performed using an M13 universal sequencing primer by a modification of the Sanger method (24).

Disruption of the *CPR1*, *CPR2*, and *CPR3* genes. Genomic DNA fragments harboring the *CPR1*, *CPR2*, and *CPR3* genes were cloned into pUC19. i) A 1.2kb SacI fragment containing the *S. cerevisiae URA3* gene (25; position -227 to +943 of the protein coding region) was inserted into the SacI site (position +104) of the 2.3kb EcoRI genomic fragment containing *CPR1*. ii) A 0.5kb ClaI fragment containing position +1 to +393 of *CPR2* was deleted from the 5.0kb EcoRI genomic fragment and replaced by a 0.85kb ClaI fragment of the *S. cerevisiae TRP1* gene (26; position -102 to +710). iii) A 1.35kb BamHI fragment containing the *S. cerevisiae HIS3* gene (27; position -447 to +880) was inserted into the XbaI site (position +52) of the 4.0kb SalI genomic fragment containing *CPR3*. Cloning manipulations were performed by standard procedures (23). DNA fragments of the disruption constructs were isolated from agarose gels and used directly to transform diploid and haploid yeast by standard methods (20).

Growth Assays. For analysis of growth on different carbon sources, cultures were

grown to mid-log phase in YPD (2.0% peptone, 1.0% yeast extract, 2.0% glucose) at 30^0C. The cells were diluted in H_2O to a density of 6.3×10^3 cells ml^{-1} and plated at a density of 6.6 cells cm^{-2} on YP (2.0% peptone, 1.0% yeast extract) containing 15 g l^{-1} Bacto agar (Difco) and either 100 mM L-lactate (Sigma), pH 4.6, 100 mM pyruvate (Sigma), pH 4.6, or 3% (v/v) glycerol. Plates were incubated at either 30^0C or 37^0C for 5 days. To prepare L-lactate- or pyruvate-containing media, the appropriate carbon source was prepared at 1M, the pH adjusted with KOH to 4.6 for L-lactate and 3.0 for pyruvate. 1 volume of the 1M carbon source was added to 1 volume of 2X YP, and the pH was readjusted to 4.6 with KOH and filter sterilized. 1 volume of the sterilized YP/carbon source mixture was added to 4 volumes YP containing 18.75 g l^{-1} Bacto agar. CsA-containing medium was prepared by dissolving CsA (a gift of Sandoz) at a concentration of 10.0 mg ml^{-1} in 83% ethanol, 17% (v/v) Tween 80 (Sigma). This mixture was diluted to 1.0 mg ml^{-1} with H_2O. 1 volume of the CsA/ethanol/Tween 80/H_2O mix was added to 9 volumes of YPD containing 16.7 g l^{-1} Bacto agar. Control plates were prepared exactly as described, but without addition of CsA. Cells were grown, diluted, and plated on medium containing or lacking CsA as described above.

Northern Blot analysis of the disrupted CPR genes. RNA isolation, agarose gel electrophoresis, transfer, hybridization, and washing were performed as described (20, 23). DNA probes were ^{32}P-labelled using a modification of the PCR method (28). Agarose gel-purified *CPR1*, *CPR2*, and *CPR3* DNA fragments (8 ng) were

each mixed in 50 μl reaction volumes containing: 200 μM each dCTP, dGTP, and dTTP; 1 μM oligonucleotide primers Y-1C (5′ AAG GGG AAA GGG GGA TCC ATG TCC CAA GTC TAT TTT GAT GTC 3′, position +1 to +24 of the predicted protein coding region) and Y-1D (5′ AAA AAA GGA GGG AAG CTT TTA TAA TTC ACC GGA CTT GGC AAC 3′, position +486 to +463) for *CPR1*; Y301 (5′ AAG GGG AAA GGG GGA TCC ATG AAA TTC AGT GGC TTG TGG TGT 3′, position +1 to +24) and Y302 (5′ AAA AAA GGA GGG GTC GAC GCA TAT ACT CAA GAA GAG AGC 3′, position +626 to +606) for *CPR2*; and Y-2A (5′ AAG GGG ATA GGG GGA TCC ACC ATG TTT AAA CGT TCC ATC ATT 3′, position +1 to +21) and Y-2B (5′ AAT TAA GGA GGG GTC GAC AAG CCT TCA TAA CTC ACC AGC TTC 3′, position +561 to +538) for *CPR3*; 1 μM [α-^{32}P]dATP (3000 Ci/mmol, 1 Ci = 37 GBq, Amersham); and 2.5 units *Thermus aquaticus* DNA polymerase (Boehringer Mannheim). 4 cycles were performed, using temperature steps of: 94^{0}C, 1.5 min.; 37^{0}C, 3.0 min.; and 72^{0}C, 2.5 min. Labelled probes were purified over Sephadex G-50 columns, and hybridized with the filter in 5 X SSC, 0.1% SDS, 50% (v/v) formamide, 5X Denhardt's solution, and 100 μg ml^{-1} sonicated, denatured salmon sperm DNA at 42^{0}C. Probes for the different CPR loci do not cross-hybridize at this stringency. The same blot was hybridized and stripped separately with each of the labeled cyclophilin PCR products.

Analysis of *CYB2* mRNA accumulation in *cpr3::HIS3* strains. Strains MB11 and MB11-3 were cultured in YPD at 30^{0}C to an A_{600} = 0.4. The cells were pelleted

by centrifugation and resuspended in either YPD or YP containing 2.0% DL-lactate to give an A_{600} = 0.2. Incubations were continued for an additional 2 hours at either 30^{0}C or 37^{0}C. RNA extraction, agarose gel electrophoresis, transfer, hybridization, and washing were performed as described. A 1,830bp *CYB2* DNA (29) probe (positions -47 to +1,783) was produced by PCR (28; primers CYB101, 5′ CGC TAA TAC AGT TCC CGC ATA 3′; and CYB102, 5′ GGC TAT AAT CAT GCA TCC TCA 3′; 20 cycles; temperature steps 94^{0}C, 1 min. 30 sec.; 55^{0}C, 2 min.; 72^{0}C, 1 min. 30 sec.), gel purified, and ^{32}P-labeled using the random hexamer method (23). The blot was stripped and rehybridized with ^{32}P-labeled *CPR1* DNA as a control for loading. *CPR1* DNA was ^{32}P-labelled by PCR as described above.

Results

We isolated two cyclophilin-related genes from a *Saccharomyces cerevisiae* genomic library by hybridization with a rat cyclophilin cDNA probe (Materials and Methods). One of these genes is *CPR1* (cyclosporin A-sensitive proline rotamase 1), one of two previously reported yeast cyclophilin genes (7, 8, 19). The other gene identified by our screen, *CPR3*, encodes a predicted protein that has 62% amino acid identity with the rat cyclophilin (22), excluding a 20 residue amino terminal leader sequence. Edmundson helical wheel projection (30) of this leader sequence suggests the formation of a positively charged, amphipathic helix similar to known mitochondrial localization signal sequences (31). The *CPR3* nucleotide sequence and comparisons of the predicted protein sequences encoded by *CPR1*, *CPR2*, and *CPR3* are presented in Figure 1A.

We inserted the *URA3*, *TRP1*, and *HIS3* genes into the protein coding regions of *CPR1*, *CPR2*, and *CPR3*, respectively, as described in Materials and Methods (Figure 1B). These disruption constructs were introduced into both diploid (CMY214) and haploid (MB11) strains. Prototrophic transformants were obtained for all three constructs (*cpr*1::*URA3*, *cpr2::TRP1*, and *cpr3::HIS3*) in both strains at approximately equal frequencies. The presence of disrupted cpr alleles in the transformants was confirmed by PCR analysis (data not shown). Northern blot analysis indicated that haploid strains harboring disruption alleles at the *CPR1*, *CPR2*, and *CPR3* loci did not produce stable mRNA of the appropriate size from the corresponding CPR genes (Figure 2). The inability of the *cpr3::HIS3*

allele to produce a truncated form(s) of the *CPR3* gene product was confirmed by Western blot analysis using a polyclonal antiserum specific for amino acid residues 82-92 of the *CPR3* gene product (data not shown). These results show that no single disruption of *CPR1*, *CPR2*, or *CPR3* affects vegetative growth at 30^0C.

We next constructed sets of isogenic strains containing all combinations of the disrupted CPR and *FPR1* (19) alleles (Table 1, *Lower*). The *FPR1* locus encodes an FK506 binding protein with PPIase activity. All of these strains are viable at 30^0C. All disruption mutants mated normally as determined by a patch mating assay. Diploids heterozygous for disruptions at all CPR loci and at *FPR1* were dissected, and all possible permutations of haploid segregants were obtained at the expected frequency for unlinked genes. Furthermore, two quadruple disruptants of opposite mating type mated normally and the resulting homozygous diploid sporulated normally. These results show that no combination of *CPR1*, *CPR2*, *CPR3*, and *FPR1* is essential for vegetative growth, mating, or sporulation at 30^0C.

A previous report stated that, whereas rho^+ yeast are resistant to CsA, rho^- yeast are sensitive to CsA at 100 μg ml^{-1} (13). Mutants of rho^- yeast resistant to CsA were reported to have decreased soluble CsA binding activity. We isolated multiple rho^- mutants of MB11, MB11-3, MB12, and MB12-1. All of these strains were resistant to CsA at 100 μg ml^{-1}.

We next tested the ability of yeast strains harboring disruption alleles of the CPR loci to grow under a variety of conditions. Strains of Table 1 were plated on YP media containing glucose, galactose, glycerol, pyruvate, or L-lactate as the sole

carbon source. The plates were incubated at either 30°C or 37°C. All strains grew on all carbon sources at 30°C. However, all *cpr3::HIS3* strains were unable to grow on L-lactate at 37°C (Figure 3). Growth of *cpr3::HIS3* strains on pyruvate at 37°C was somewhat depressed. Disruption of the other CPR loci and *FPR1*, alone or in combination, had no effect on growth on L-lactate at 37°C. The defect is recessive as *CPR3/cpr3::HIS3* diploids grow on L-lactate at 37°C, while *cpr3::HIS3/cpr3::HIS3* diploids do not.

In the oxidation of L-lactate to pyruvate in the intermembrane space of *S. cerevisiae* mitochondria, flavocytochrome b_2 catalyzes the transfer of two electrons from L-lactate to cytochrome c (32). No other pathway is used by *S. cerevisiae* for L-lactate metabolism (29). Although the growth of *cpr3::HIS3* strains is slightly depressed on pyruvate at 37°C, the absolute requirement of *CPR3* for growth on L-lactate at 37°C indicates that the *CPR3* gene product may be involved in a stage of L-lactate metabolism prior to its oxidation to pyruvate. Furthermore, the ability of *cpr3::HIS3* strains to grow on glycerol and pyruvate indicates that the *CPR3* gene product is not essential for general mitochondrial function.

Transcription of *CYB2*, the gene encoding flavocytochrome b_2, is repressed by growth on glucose and induced by growth on non-fermentable carbon sources (29). We analyzed the induction of *CYB2* mRNA in strains MB11 and MB11-3 at either 30°C or 37°C (Figure 4). Both strains produce stable *CYB2* mRNA at either temperature, indicating that the *CPR3* gene product is not required for *CYB2* mRNA induction or stability.

Discussion

We have isolated and characterized a yeast cyclophilin gene, *CPR3*, which probably encodes a mitochondrial isoform, and thus may be the first gene identified to encode a distinct mitochondrial cyclophilin. The mitochondrial cyclophilin from *Neurospora crassa* is generated by alternate splicing of a single cyclophilin gene transcript that also encodes a cytosolic isoform (6).

Disruption of the *CPR3* gene causes a temperature sensitive defect in L-lactate metabolism. Two questions are raised by the appearance of this phenotype. First, what aspect of L-lactate metabolism is affected? Second, why is the phenotype temperature sensitive? The defect is recessive and specific for growth on L-lactate, as *CPR3* disruption mutants are capable of growth at 37^0C on glycerol and, albeit at a reduced rate, on pyruvate. The poor growth of *cpr3::HIS3* mutants on pyruvate is consistent either with a separate role of *CPR3* in pyruvate metabolism, or with roles for *CPR3* on a process(es) specific for L-lactate metabolism, as well as on a process(es) shared by L-lactate and pyruvate metabolism.

Our data show that the *CPR3* gene product is not required for the induction or stability of *CYB2* mRNA. One possibility for the *CPR3*-requiring process is the maturation or post-translational stability of flavocytochrome b_2. Biochemical experiments to assess flavocytochrome b_2 activity in *CPR3* disruption mutants remain to be done.

The temperature dependence of the defect is consistent with a role for the

CPR3 gene product in protein folding. The temperature-sensitive defect is reminiscent of the requirement for heat shock proteins in mitochondrial biogenesis (33, 34). Perhaps maturation of flavocytochrome b_2, and of other mitochondrial proteins, has a dependence on helper functions such as PPIase at higher temperature. While we have not yet demonstrated PPIase activity for the *CPR3* gene product, the *CPR3* gene expressed in E. coli encodes a CsA binding activity (E.S.D. and M.B.B., unpublished observations) and the *CPR3* predicted protein sequence has high sequence identity with cyclophilins known to have PPIase activity.

One model consistent with our data is that the *CPR3* gene product is involved in the folding or refolding of flavocytochrome b_2. Should the *CPR3* gene product be located in the intermembrane space, either explanation would be possible. However, if the *CPR3* gene product is located in the mitochondrial matrix, then the explanation may be less straight forward, possibly involving transport of the flavocytochrome b_2 precursor. We have isolated both dominant and recessive extragenic suppressors of *cpr3::HIS3* strains. These mutations allow growth on L-lactate at 37^0C. Characterization of these suppressors may identify substrates or coeffectors of the *CPR3* gene product.

References

1. Dreyfuss, M., Harri, E., Hofmann, H., Kobel, H., Pache, W., & Tscherter, H. (1976) *Eur. J. Appl. Microb.* **3**, 125-133.

2. Handschumacher, R. E., Harding, M. W., Rice, J., Drugge, R. J., & Speicher, D. W. (1984) *Science* **226**, 544-547.

3. Haendler, B., Hofer-Warbinek, R., & Hofer, E. (1987) *EMBO J.* **6**, 947-950.

4. Shieh, B-H., Stamnes, M. A., Seavello, S., Harris, G. L., & Zuker, C. S. (1989) *Nature* **338**, 67-70.

5. Stamnes, M. A., Shieh, B-H., Chuman, L., Harris, G. L., & Zuker, C. S. (1991) *Cell* **65**, 219-227.

6. Tropschug, M., Nicholson, D. W., Hartl, F-U., Kohler, H., Pfanner, N., Wachter, E., & Neupert, W. (1988) *J. Biol. Chem.* **263**, 14433-14440.

7. Haendler, B., Keller, R., Hiestand, P. C., Kocher, H. P., Wegmann, G., & Movva, N. R. (1989) *Gene* **83**, 39-46.

8. Koser, P. L., Bergsma, D. J., Cafferkey, R., Eng, W-K., McLaughlin, M. M.,

Ferrara, A., Silverman, C., Kasyan, K., Bossard, M. J., Johnson, R. K., Porter, T. G., Levy, M. A., Livi, G. P. (1991) *Gene* **108**, 73-80.

9. Hasel, K. W., Glass, J. R., Godbout, M., & Sutcliffe, J. G. (1991) *Mol. Cell. Biol.* **11**, 3484-3491.

10. Spik, G., Haendler, B., Delmas, O., Mariller, C., Chamoux, M., Maes, P., Tartar, A., Montreuil, J., Stedman, K., Kocher, H. P., Keller, R., Hiestand, P. C., & Movva, N. R. (1991) *J. Biol. Chem.* **266**, 10735-10738.

11. Caroni, P., Rothenfluh, A., McGlynn, E., & Schneider, C. (1991) *J. Biol. Chem.* **266**, 10739-10742.

12. Liu, J., & Walsh, C. T. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4028-4032.

13. Tropschug, M., Barthelemess, I. B., & Neupert, W. (1989) *Nature* **342**, 953-955.

14. Friedman, J., & Weissman, I. (1991) *Cell* **66**, 799-806.

15. Liu, J., Farmer, Jr., J. D., Lane, W. S., Friedman, J., Weissman, I., & Schreiber, S. L. (1991) *Cell* **66**, 807-815.

16. Takahashi, N., Hayano, T., & Suzuki, M. (1989) *Nature* **337**, 473-4751.

17. Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., & Schmid, F. X. (1989) *Nature* **337**, 476-478.

18. Gething, M-J., & Sambrook, J. (1992) *Nature* **355**, 33-45.

19. Heitman, J., Movva, N. R., Hiestand, P. C., & Hall, M. N. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1948-1952.

20. Sherman, F., Fink, G. R., & Hicks, J. B. Laboratory Course Manual for Methods in Yeast Genetics (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986).

21. Hochgeschwender, U., Sutcliffe, J. G., & Brennan, M. B. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8482-8486.

22. Danielson, P. E., Forss-Petter, S., Brow, M. A., Calavetta, L., Douglass, J., Milner, R. J., & Sutcliffe, J. G. (1988) *DNA* **7**, 261-267.

23. Sambrook, J., Fritsch, E. F., & Maniatis, T. Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).

24. Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463-5467.

25. Rose, M., Grisafi, P., & Botstein, D. (1984) *Gene* **29**, 113-124.

26. Tschumper, G. & Carbon, J. (1980) *Gene* **10**, 157-166.

27. Struhl, K. (1985) *Nucleic Acids Res.* **13**, 8587-8601.

28. Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., & Arnheim, N. (1985) *Science* **230**, 1350-1354.

29. Guiard, B. (1985) *EMBO J.* **4**, 3265-3272.

30. Schiffer, M. & Edmundson, A. B. (1967) *Biophys. J.* **7**, 121-135.

31. Attardi, G. & Schatz, G. (1988) *Ann. Rev. Cell Biol.* **4**, 289-333.

32. Appelby, C. A. & Morton, R. K. (1954) *Nature* **173**, 749-752.

33. Cheng, M. Y., Hartl, F-U., Martin, J., Pollock, R. A., Kalousek, F., Neupert, W., Hallberg, E. M., Hallberg, R. L., & Horwich, A. L. (1989) *Nature* 337, 620-625.

34. Scherer. P. E., Krieg, U. C., Hwang, S. T., Vestweber, D., & Schatz, G. (1990) *EMBO J.* **9**, 4315-4322.

Table 1. Yeast Strains.

A. Triple disruption series derived from MB11.

Strain	Genotype
MB11	*MATα ade2-101 trp1-Δ1 ura3-52 his3-Δ200 lys2-801 can1*
MB11-3	MB11 *cpr3::HIS3*
MB12	MB11 *cpr1::URA3*
MB12-1	MB11 *cpr1::URA3 cpr3::HIS3*
ED19	MB11 *cpr2::TRP1*
ED20	MB11 *cpr2::TRP1 cpr3::HIS3*
ED21	MB11 *cpr1::URA3 cpr2::TRP1*
ED22	MB11 *cpr1::URA3 cpr2::TRP1 cpr3::HIS3*

B. Quadruple disruption series derived from MH272-3c.

Strain	Genotype
MH272-3c	*MAT***a** *trp1-1 his3 leu2-3,112 ura3-52 HML***a**
MH274-1a	MH272-3c *fpr1::URA3*
MH274-2d	MH272-3c *cpr1::LEU2 fpr1::URA3*
MH274-4a	MH272-3c *cpr1::LEU2*
ED80-1	MH272-3c *cpr3::HIS3*
ED81-1	MH272-3c *cpr1::LEU2 cpr3::HIS3*

Table 1B (continued).

Strain	Genotype
ED82-2	MH272-3c *cpr2::TRP1 fpr1::URA3*
ED83-1	MH272-3c *cpr1::LEU2 cpr2::TRP1 fpr1::URA3*
ED84-1	MH272-3c *cpr1::LEU2 cpr2::TRP1*
ED85-1	MH272-3c *cpr1::LEU2-1 cpr3::HIS3 fpr1::URA3*
ED86-1	MH272-3c *cpr1::LEU2-1 cpr2::TRP1 cpr3::HIS3 fpr1::URA3*
ED87-1	MH272-3c *cpr2::TRP1 cpr3::HIS3*
ED88-1	MH272-3c *cpr3::HIS3 fpr1::URA3*
ED89-1	MH272-3c *cpr2::TRP1 cpr3::HIS3 fpr1::URA3*
ED90-1	MH272-3c *cpr1::LEU2 cpr2::TRP1 cpr3::HIS3*
ED91-1	MH272-3c *cpr2::TRP1*

Figure 1. a) Sequence analysis of the *Saccharomyces cerevisiae CPR3* gene. The *CPR3* nucleotide sequence and the predicted protein sequences of the yeast *CPR1*, *CPR2*, and *CPR3* genes are aligned. A yeast "consensus sequence" is included: Residues conserved among all three yeast cyclophilin proteins are indicated in capital letters, while lower case letters refer to residues common to two of the three proteins. b) Structure of the disrupted CPR genes. Black boxes represent protein coding regions of the CPR genes. Flanking solid lines represent non-coding sequences. E, Eco RI; S, Sac I; C, Cla I; L, Sal I; X, Xba I.

Figure 1A.

```
CPR3 DNA                                          tacttaccATGTTTAAACGTTCCATCATTCAACAGTCCCGCTTATTCTCCAATTCTGCTTCTAGACTAGGTAAA   -8-66
CPR3 Prot                                       M F K R S I I Q Q S R L F S N S A S R L G K   1-22
CPR1 Prot                                                                                 M S   1-2
CPR2 Prot  M K F S G L W C W L L L F L S V N V I A S D V G E L I D Q D D E V I T Q   1-36
Consensus  - - - - - - - - - - - - - - - - - - - - - - - - - l - - - - - - - - - -
CPR3 DNA   AAAGTGTTCTTTGATCCTGCGGTCAATGGAACCAAAATCGGCAGAATAGAATTTGAATTATATGATAATGTGGTTCCTAAAACTGCTGAAAATTTCAGAGCCTTATGT   67-174
CPR3 Prot  K V F F D P A V N G T K I G R I E F E L Y D N V V P K T V E N F R A L C   23-58
CPR1 Prot  Q V Y F D V E A D G Q P I G R V V F K L Y N D I V P K T A E N F R A L C   3-38
CPR2 Prot  K V F F D I E H G E E K V G R I V I G L Y G K V C P K T A K N F Y K L S   37-72
Consensus  k V f F D - e - - g - k i G R i v f - L Y - - v v P K T a e N F r a L c
CPR3 DNA   ACCGGCGAAAAGGGCTGG---GGCTATAAAGGTGTCCCTTTCCACAGAATCATCCCAGACTTCATGATCCAAGGTGGTGACACTGATTTGACTAACGGTTTTGGGGGC   175-279
CPR3 Prot  T G E K G W - G Y K G V P F H R I I P D F M I Q G G D T D L T N G F G G   59-93
CPR1 Prot  T G E K G F - G Y A G S P F H R V I P D F M L Q G G D F T A G N G T G G   39-73
CPR2 Prot  T T T N S K K G F I G S T F H R V I P N F M V Q G G D F T D G T G V G G   73-108
Consensus  T g e k g - - G y - G s p F H R v I P d F M - Q G G D f t - g n G - G G
CPR3 DNA   AAATCCATTTACGGTTCCAAGTTCGCTGACGAAAATTTTGTCAAAAAGCATGACAAGGCAGGTTTGTTATCTATGGCAAATGCTGGACCAAACACTAACGGTTCTCAA   280-387
CPR3 Prot  K S I Y G S K F A D E N F V K K H D K A G L L S M A N A G P N T N G S Q   94-129
CPR1 Prot  K S I Y G G K F P D E N F K K H H D R P G L L S M A N A G P N T N G S Q   74-109
CPR2 Prot  K S I Y G D T F P D E N F T L K H D R K G R L S M A N R G K D T N G S Q   109-144
Consensus  K S I Y G - k F p D E N F - k k H D r - G l L S M A N a G p n T N G S Q
CPR3 DNA   TTCTTCATTACCACTGTGCCTTGTCCA---TGGTTGGATGGAAAACACGTTGTCTTTGGTGAGGTAACCAAAGGTATGGACATTGTCAAAGCAATCGAATCATACGGT   388-492
CPR3 Prot  F F I T T V P C P - W L D G K H V V F G E V T K G M D I V K A I E S Y G   130-164
CPR1 Prot  F F I T T V P C P - W L D G K H V V F G E V V D G Y D I V K K V E S L G   110-144
CPR2 Prot  F F I T T T E E A S W L D G K H V V F G Q V V D G M D V V N Y I Q H V S   145-180
Consensus  F F I T T v p c p - W L D G K H V V F G e V v d G m D i V k - i e s - g
CPR3 DNA   ACTGCTTCTGGTAAACCAAGAGCTGAAATCGTT---ATCGAAGAAGCTGGTGAGTTAtgaaggcttatatgaagctaaggtctctcatatttctag   493-585
CPR3 Prot  T A S G K P R A E I V - I E E A G E L - - - - - -   165-182
CPR1 Prot  S P S G A T K A R I V - V A K S G E L - - - - - -   145-162
CPR2 Prot  R D A N D K P L E A V K I A K C G E W T P E L S S   181-205
Consensus  - - s g - - - a e i V - i a k - G E l - - - - - -
```

Figure 1B.

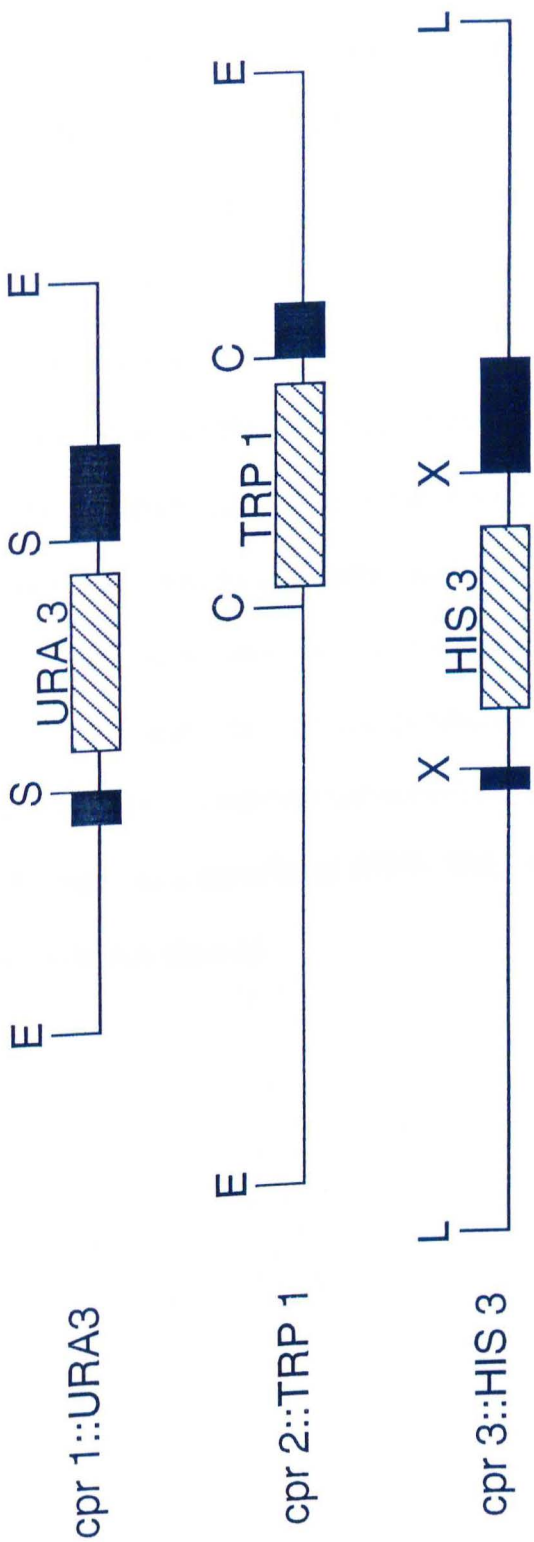


Figure 2. Northern blot analysis of the disrupted CPR genes. Lanes 1, MB11 (*CPR1 CPR2 CPR3*); 2, MB11-3 (*CPR1 CPR2 cpr3::HIS3*); 3, MB12 (*cpr1::URA3 CPR2 CPR3*); 4, MB12-1 (*cpr1::URA3 CPR2 cpr3::HIS3*); 5, ED19 (*CPR1 cpr2::TRP1 CPR3*); 6, ED20 (*CPR1 cpr2::TRP1 cpr3::HIS3*); 7, ED21 (*cpr1::URA3 cpr2::TRP1 CPR3*); and 8, ED22 (*cpr1::URA3 cpr2::TRP1 cpr3::HIS3*). The same blot was hybridized separately with ^{32}P labeled *CPR1*, *CPR2*, and *CPR3* DNA, as indicated. Disruption alleles at the *CPR1* and *CPR3* loci lead to the production of mRNA of an inappropriate size. The smaller size mRNAs produced by the *cpr1::URA3* and *cpr3::HIS3* alleles probably result from premature transcription termination in the inserted sequences. The *cpr2::TRP1* allele harbors a 5′ terminal deletion of 393 base pairs of the predicted protein coding region of *CPR2*, and is thus incapable of initiating transcription. In whole cell extracts, only disruption of *CPR1* leads to a significant (60%-70%) loss of soluble cyclosporin A binding activity (data not shown).

Figure 2.

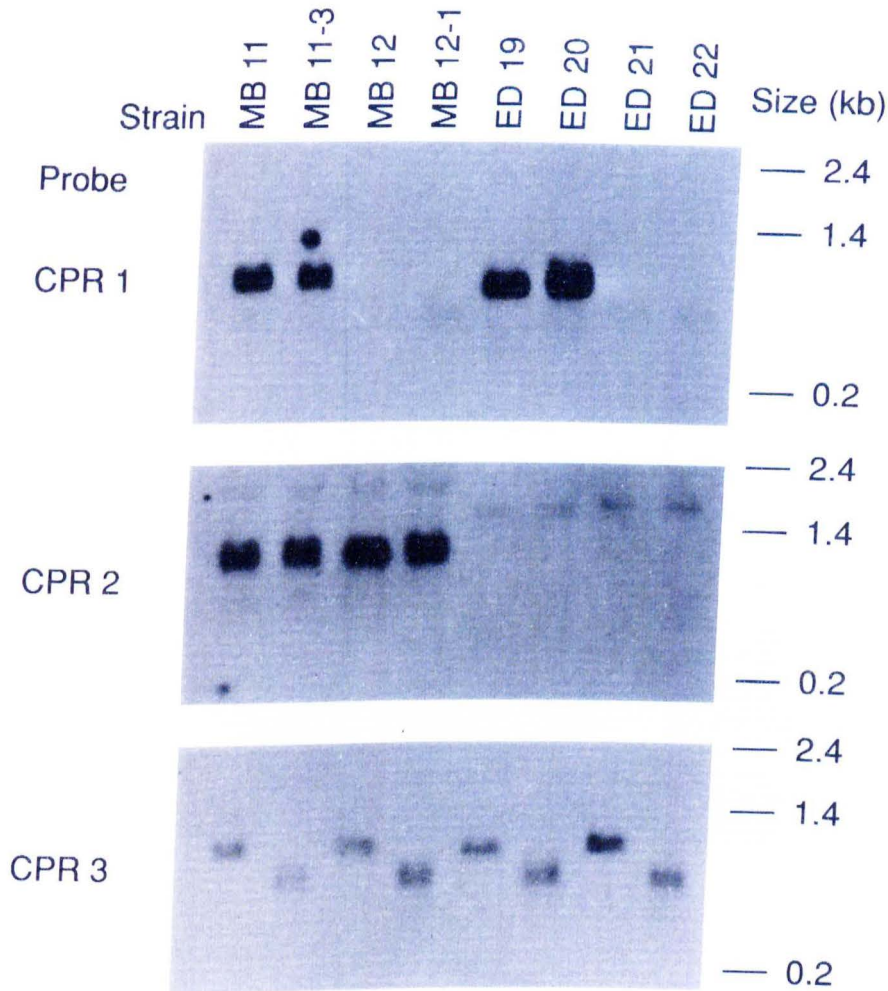

Strain
MB 11
MB 11-3
MB 12
MB 12-1
ED 19
ED 20
ED 21
ED 22
Size (kb)
Probe
CPR 1
2.4
1.4
0.2
CPR 2
2.4
1.4
0.2
CPR 3
2.4
1.4
0.2

Figure 3. Analysis of MB11 (*CPR3*) and MB11-3 (*cpr3::HIS3*) growth at 30^0C and 37^0C on L-lactate (L); pyruvate (P); and glycerol (G).

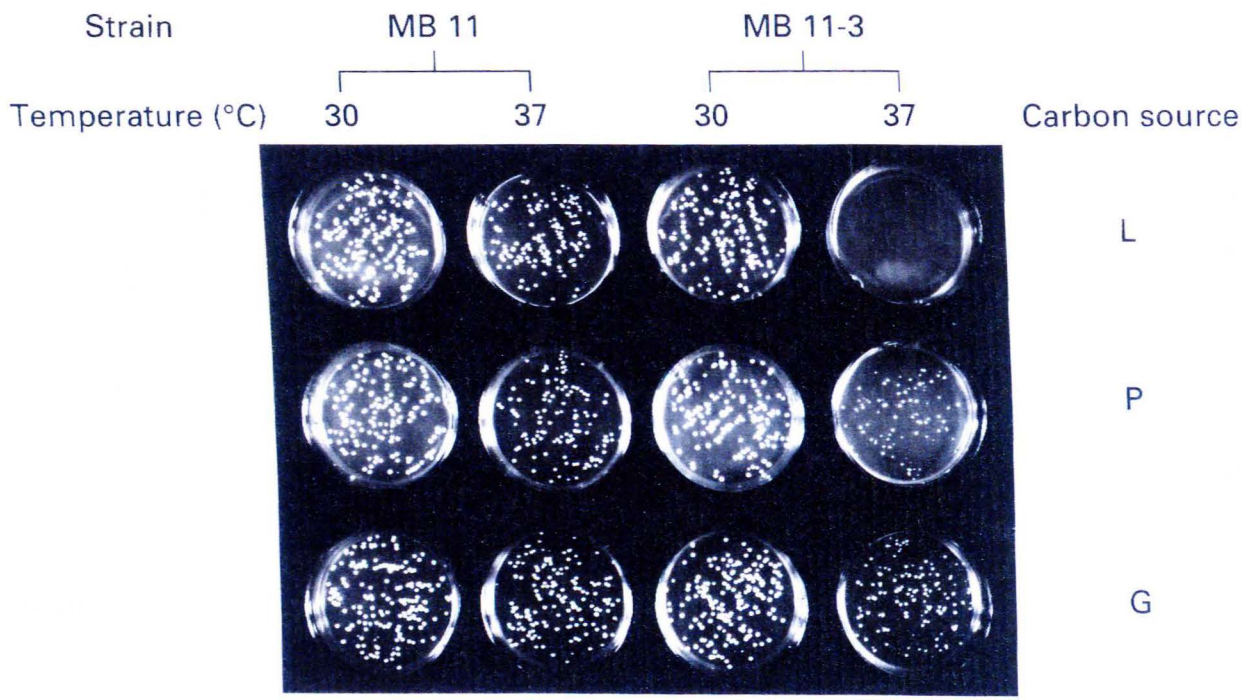
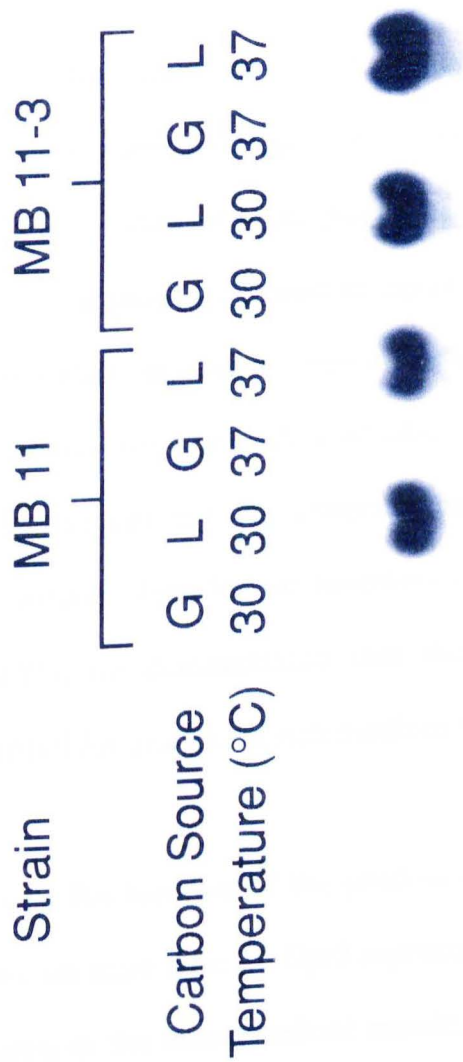

Strain
MB 11
MB 11-3
Temperature (°C)
30
37
30
37
Carbon source
L
P
G

Figure 3.

Figure 4. Effect of *CPR3* disruption on *CYB2* mRNA accumulation at 30^0C and 37^0C. Northern blot of RNA from strains MB11 (*CPR3*) and MB11-3 (*cpr3::HIS3*), hybridized with ^{32}P labelled *CYB2* DNA (29). Strains were incubated in the presence of either glucose (G) or DL-lactate (L), at 30^0C or 37^0C.

Figure 4.


Strain
MB 11
MB 11-3
Carbon Source G L G L G L G L
Temperature (°C) 30 30 37 37 30 30 37 37

Chapter 3. Cpr3 Biochemistry: The Yeast Cyclophilin Cpr3 is a Mitochondrial Matrix Protein Important for Mitochondrial Function.

Summary

The cyclophilins are a family of eukaryotic proteins that mediate the biological actions of the immunosuppressant drug, cyclosporin A (CsA). While the normal physiological functions of cyclophilins are unknown, their participation in immunosuppression suggests a function in signal transduction pathways. *In vitro*, cyclophilins have a CsA-sensitive proline isomerase (PPIase) activity, suggesting that they may catalyze protein folding *in vivo*. However, inhibition of PPIase activity does not account for the observed pharmacological effects of CsA; elucidating the normal physiological functions of cyclophilins may resolve this paradox. Recently, we demonstrated that the cyclophilin gene *CPR3* of *S. cerevisiae* is essential for growth on rich medium supplemented with lactic acid at 37^0C.

In dissecting the function of the product of the *CPR3* gene, Cpr3, at the biochemical level, we show that: 1. Cpr3 expressed in *Escherichia coli* binds CsA; 2. Cpr3 is localized in the mitochondrial matrix; 3. Disruption of *CPR3* has no effect on the expression of mRNA for two genes reported to be required for growth on lactate, *CYB2* (gene for flavocytochrome b_2, a mitochondrial lactate dehydrogenase), and *CYC1* (gene for iso-1-cytochrome c). 4. Inhibition of flavocytochrome b_2 activity is insufficient to explain the phenotype of *cpr3::HIS3*

mutants. We conclude that the Cpr3 function is not necessary for signal transduction controlling cytochrome gene expression. The physiological role of Cpr3 is not limited to lactate metabolism, but plays a general role in mitochondrial function.

Introduction

Cyclophilins were first identified as two soluble 17 kd proteins from bovine thymus that bind the immunosuppressive drug cyclosporin A (CsA; Handschumacher, *et al.*, 1984). Cyclophilins comprise a large family of highly conserved, abundant proteins found in all eukaryotes examined (Koltsky, *et al.*, 1986). The normal physiological functions of cyclophilins are unknown.

Cyclophilins probably mediate the pharmacological activities of CsA, an undecameric, hydrophobic peptide metabolite of the fungus *Tolypocladium inflatum*. CsA is cytotoxic to most eukaryotes (Borel, *et al.*, 1976, 1977), and is an immunosuppressant, used to prevent the rejection of organ and tissue transplants (Borel, *et al.*, 1976). CsA blocks a Ca^{2+}-dependent step in the activation of antigen- and mitogen-stimulated T-helper lymphocytes, by interfering with the assembly of NFAT, a transcriptional activator of lymphokine genes (Krönke, *et al.*, 1984; Emmel, *et al.*, 1989; Flanagan, *et al.*, 1991).

In vitro, CsA binding to cyclophilin inhibits the activity of calcineurin, a serine/threonine protein phosphatase (Liu, *et al.*, 1991). Inhibition of calcineurin is probably relevant to the immunosuppressive action of CsA, as overexpression of calcineurin decreases the CsA-sensitivity of the IL-2 promoter (Clipstone and Crabtree, 1992; O'Keefe, *et al.*, 1992). CsA probably does not exert its biological effects by inhibiting cyclophilin function, as CsA-resistant fungal mutants have lost immunologically detectable cyclophilin and CsA binding activity (Tropschug, *et al.*, 1989). Instead, CsA most likely affects signal transduction by forming a dominant

inhibitory complex with cyclophilin (Tropschug, *et al.*, 1989).

Cyclophilins possess peptidyl-prolyl *cis-trans* isomerase (PPIase) activity *in vitro*, which is inhibited by CsA (Fischer, *et al.*, 1989; Takahashi, *et al.*, 1989). PPIase accelerates the rate of interconversion of *cis* and *trans* isomers of prolyl amide bonds in some small peptides and proteins *in vitro*. Although proline isomerization *in vitro* can occur spontaneously and rapidly (Fischer, et. al, 1989), it is possible that, *in vivo*, the cyclophilin PPIase activity might play roles in assisted protein folding.

The *ninaA* gene product of *Drosophila melanogaster* is a cyclophilin homolog essential for the post-transcriptional production of Rh1 rhodopsin in the secretory pathway of photoreceptor cells (Shieh, *et al.*, 1989; Colley, *et al.*, 1991). This finding is consistent with a role for cyclophilins in protein folding, although there is no biochemical or genetic evidence that the *ninaA* gene product interacts directly with Rh1 rhodopsin. Further, it is not yet known whether the *ninaA* gene product has PPIase activity.

The mechanism of CsA action in immunosuppression suggests a physiological role of cyclophilins in signal transduction, whereas the PPIase activity suggests that cyclophilins catalyze protein folding. However, a synthetic analog of CsA inhibits cyclophilin PPIase activity, but is not immunosuppressive (Sigal, *et al.*, 1991). By determining the normal physiological roles of cyclophilin, it may be possible to resolve the apparently paradoxical involvement of cyclophilins in signal transduction and protein folding.

We have previously reported that disruption of one of the four known yeast

cyclophilin genes, *CPR3*, results in the inability of yeast to grow on rich medium supplemented with L-lactic acid at 37^0C (Davis, *et al.*, 1992). To understand the basis of this phenotype, it is necessary to study the gene product, especially its location in the cell, and to determine the biochemical nature of the defect.

Here we show that Cpr3 is a CsA binding protein located in the mitochondrial matrix. Further, we show that *cpr3::HIS3* strains are not deficient in the signal transduction pathway necessary for regulating cytochrome gene expression. Finally, the phenotype resulting from loss of Cpr3 is not caused by a deficiency in flavocytochrome b_2, a mitochondrial lactate dehydrogenase. We conclude that Cpr3 plays a general role in mitochondrial function, but is not necessary for signal transduction.

Experimental procedures

Yeast strains and media

Strains MB11, MB11-3, MB12, MB12-1, ED19, ED20, ED21, and ED22 were described previously (Davis, *et al.*, 1992). Strains were normally cultured in YPD (2.0% peptone, 1.0% yeast extract, 2.0% glucose; Sherman, *et al.*, 1986) at 30^0C. Cell growth was tested on YP containing either 2.0% (w/v) D-, L-lactate, 3.0% glycerol, or without added carbon source. Unsupplemented medium was pH-adjusted to 4.6 with HCl and KOH. D-, L-lactate was initially prepared at 20%, pH 4.6 with KOH, filter-sterilized, and added to autoclaved 1.1 X YP to a final concentration of 2.0%. For plates, 15 g L^{-1} agar was included.

Western analysis of Cpr3

Strains MB11, MB11-3, MB12, and ED19 (Table 1) were grown in liquid YP medium containing 3.0% glycerol to an A_{600} = 0.3. Cells were pelleted and lysed with glass beads as described (Harlow & Lane, 1988) in RIPA (150 mM NaCl, 1.0% nonidet-P40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl pH 8.0, 2 μg ml^{-1} aprotinin, 2mM EDTA, 2 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin, and 100 μg ml^{-1} phenyl methane sulfonyl fluoride). Proteins were separated on 15% gels by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and transferred to polyvinyl difluoride (PVDF) or nitrocellulose membranes using standard methods (Harlow & Lane, 1988). Duplicate gels were stained with Coomassie brilliant blue (Sigma) as described (Harlow and Lane, 1988).

Cpr3-specific peptides cpr3-1 (N-Gly-Thr-Ala-Ser-Gly-Lys-Pro-Arg-Ala-Glu-C, positions 164-173) and cpr3-2 (N-Gly-Asp-Thr-Asp-Leu-Thr-Asn-Gly-Phe-Gly-C, positions 83-92) were synthesized by Synthecell (Rockville, MD). Rabbit polyclonal antisera (Berkeley Antibody Company) were raised against peptides coupled to keyhole limpet hemocyanin. Immunoprobing was performed according to standard protocols (Harlow and Lane, 1988). Following transfer, membranes were washed once for 5 minutes at room temperature in Tris Buffered Saline (TBS; 0.025M Tris-HCl, pH 8.0, 0.137M NaCl, 0.003M KCl) containing 0.2% (v/v) Tween 20. Next, membranes were washed four times for 5 min. each at room temperature in TBS. Non-specific sites were blocked by incubating membranes for 2 hr. at room temperature in blocking buffer (TBS containing 3.0% bovine serum albumin and 0.02% NaN_3). Membranes were washed twice in TBS, and then incubated for 1 hour with blocking buffer containing 1 X 10^{-3} dilutions of the appropriate, whole antisera. Membranes were washed four times in TBS and incubated for 1 hour in blocking buffer containing a 0.5 X 10^{-3} dilution of goat anti-rabbit IgG conjugated to bacterial alkaline phosphatase (Sigma). After four additional washes in TBS, protein bands were visualized by incubating the membranes in 0.1 M Tris-HCl, (pH 9.5), 0.1 M NaCl, 0.005M $MgCl_2$, 0.0004M nitroblue tetrazoleum, and 0.0004M 5-bromo, 4-chloro, 3-indolylphosphate as described (Harlow & Lane, 1988).

Subcellular localization

Mitochondria were isolated from spheroplasts and fractionated by a

modification of the differential centrifugation method of Daum and Schatz (1982). Strain MB11 (Table 1) was grown in 0.5 L of YP containing 3.0% glycerol in each of four 2L Erlenmeyer flasks to an A_{600} = 0.5. An aliquot of broken spheroplasts was saved as the "whole-cell" fraction. The remainder was centrifuged at 4^0C in a Beckman JA-20 rotor at 9000 rpm for 10 min. An aliquot of the supernatant was saved as the "cytosolic" fraction. The pellet was gently resuspended in the supernatant and diluted with 137 ml of homogenization buffer. Whole mitochondria, intermembrane space, and matrix proteins were then separated from membranes as described (Daum, *et al.*, 1982). SDS-PAGE and Western blot analysis with antiserum Abcpr3-1 were performed as described above. Enzyme assays for flavocytochrome b_2 (Labeyrie, *et al.*, 1978) and for fumarase (Racker, 1950) were performed as described (see below).

Cyclosporin A binding assays

Yeast strains were grown in YPD (2.0% peptone, 1.0% yeast extract, 2.0% glucose) to an A_{600} of 0.2-0.4. Cells were pelleted by centrifugation, washed once with 1.0M sorbitol, and resuspended in spheroplast buffer (0.9M sorbitol, 0.05M sodium phosphate, pH 7.5, 0.014M 2-mercaptoethanol), containing 50 u ml^{-1} lyticase (Sigma). After incubation at 30^0C for 30 min., the cells were washed twice with 1.0M sorbitol, and resuspended in 0.02M Tris-HCl, pH 7.5, 0.005M ß-mercaptoethanol. The cells were disrupted by sonication for 20 sec. on ice with a Vibra-Cell microprobe (80% maximum output). Protein concentrations were determined by the method of Bradford (1976). Unlabeled CsA was a gift of

Sandoz. ^{3}H-CsA was purchased from Amersham. CsA binding assays were performed by Sephadex LH-20 column fractionation of mixtures of crude extracts and ^{3}H-CsA as described (Handschumacher, *et al.*, 1984).

Expression of Cpr1 and Cpr3 in Escherichia coli

CPR1, *CPR3FL*, and *CPR3Δ* were synthesized from yeast genomic DNA (strain MB11) using polymerase chain reaction (PCR) amplification. Approximately 10 ng of DNA was mixed in 100 μl volumes with 200 μM each dCTP, dGTP, dTTP, and dATP; 1μM each oligonucleotide primer: Y1-5 (5′ AGG CTG GAT CCA TGT CCC AAG TCT ATT TTG A 3′; positions +1 to +20 of the protein coding region) and Y1-3 (5′ ACG TAG CAT GCA AGC GTT ATA ATT CAC CGG A 3′; positions +494 to +475) for *CPR1*; Y2-5+ (5′ AGG CTG GAT CCT TAC CAT GTT TAA ACG TTC C 3′; positions -5 to +15) and Y2-3 (5′ AGG TAG CAT GCA GCC TTC ATA ACT CAC CAG C 3′; positions +553 to +535) for *CPR3FL*; and Y2-5- (5′ AGG CTG GAT CCA TGA AAG TGT TCT TTG ATC CTG C 3′; positions +67 to +86 of *CPR3*) and Y2-3 for *CPR3Δ*; and 5 units *T. aquaticus* DNA polymerase (Boehringer Mannheim). 40 cycles were performed, consisting of 94^{0}C, 1 min. 30 sec; 50^{0}C, 3 min.; and 72^{0}C, 2 min. 30 sec. PCR products were digested with restriction endonucleases Bam HI and SphI, gel-purified, and cloned into Bam HI, SphI-digested plasmid pNH18a (Hasan and Szybalski, 1987) by standard methods (Sambrook, *et al.*, 1989). The recombinant clones were transformed into *Escherichia coli* strain D1210HP (*lacI*^{q}, λ *Int*$^{+}$ *Xis*$^{-}$ *Kil*$^{-}$; Hasan and Szybalski, 1987), and grown in Luria-Bertrani (LB)

medium containing 50 μg ml^{-1} ampicillin at 30^{0}C. To induce unidirectional, *Int*-dependent inversion of the *plac* promoter to the "ON" phase, an equal volume of LB medium pre-warmed to 55^{0}C was added, and cultures were incubated at 42^{0}C for 10 min. Cultures were then incubated at 30^{0}C for 3 hours. *plac* promoter inversion was confirmed by restriction enzyme analysis of isolated plasmid DNA.

Induction of *plac*-mediated expression was performed essentially as described (Hasan and Szybalski, 1987). Cultures containing "ON"-phase plasmids were grown in 50 ml LB containing 50μg ml^{-1} ampicillin to an A_{600} = 0.6. One-half of each culture was diluted 2-fold in LB medium containing isopropyl ß, D-thiogalactopyranoside (IPTG) to a final concentration of 0.001M. As a control to determine background CsA-binding activity, the other one-half was diluted in LB lacking IPTG. Incubation was continued at 30^{0}C for 3 hours. Whole cell protein extracts were prepared by a method from J. Kobori (personal communication): Cells were pelleted by centrifugation and resuspended in 80 μl of buffer containing 0.05M Tris-HCl, pH 7.4, 10% (w/v) sucrose. Cells were frozen at -80^{0}C, thawed, and placed on ice. 7 μl of 0.25M Tris-HCl, pH 7.4, containing 10mg/ml lysozyme was added to the cells, and incubations continued on ice for 45 min. Extracts were microcentrifuged at 4^{0}C for 20 min at 14,000 rpm. The pellets were discarded, and protein concentrations were determined by the method of Bradford (1976). CsA binding assays were performed as described above (Handschumacher, *et al.*, 1984).

Analysis of flavocytochrome b_2 activity in whole cell extracts

Strains MB11 and MB11-3 were cultured in YP containing 10% glucose at 30^0C to an A_{600} = 0.2-0.9. The cells were pelleted by centrifugation and resuspended in 0.5 ml H_2O. Cells were added to 50 ml YP containing either 3.0% glycerol or 2.0% D-, L-lactate in baffled flasks, to give an A_{600} = 0.03. The medium was pre-warmed to either 30^0C or 37^0C. Incubations were continued with 300 rpm shaking for the indicated time and temperature. Cells were pelleted, and whole cell extracts were prepared using glass beads as described (Guiard, 1985). Protein concentrations were determined by the method of Bradford (1976). Flavocytochrome b_2 activity in whole cell extracts was determined as described (Labeyrie, *et al.*, 1978) by spectrophotometric measurement of potassium ferricyanide reduction. Proteins were added to reaction cocktails pre-warmed to 30^0C in a water-jacketed spectrophotometer cuvette (Pharmacia). Reactions (0.6 ml final volume) consisted of 0.1M sodium phosphate, pH 7.0, containing 0.01M L-lactate (Sigma), 0.001M potassium ferricyanide (Sigma). The decrease in absorbance at 420 nm at 30^0C was recorded every 15 seconds for 4 minutes. Background was determined in duplicate reactions without L-lactate. One unit of flavocytochrome b_2 is equal to the transfer of 1 μmol electron equivalent per minute at 30^0C (Labeyrie, *et al.*, 1978). Fumarase assays were performed as described (Racker, 1950). Proteins were added to reaction cocktails pre-warmed to 30^0C in a water-jacketed spectrophotometer cuvette (Pharmacia). Reactions (0.6 ml final volume) consisted of 0.05M potassium phosphate, pH 7.4, 0.05M sodium L-malate. The increase in absorbance at 240 nm at 30^0C was recorded every 15 seconds for 4 minutes. Background was determined in duplicate

reactions without sodium L-malate. One unit is equal to a change of the A_{240} of 0.001 per minute (Racker, 1950).

Analysis of CYB2, CYC1, *and* ACT1 *mRNA induction*

Strains MB11 and MB11-3 were cultured in YP containing 10% glucose at 30^0C to an A_{600} = 0.2-0.9. One aliquot was removed for RNA preparation. Cells of another aliquot were pelleted by centrifugation and resuspended in 2.0 ml H_2O. Cells were diluted in YP containing 2.0% D-, L-lactate, pH 4.6 in baffled flasks, to give an A_{600} = 0.03. The medium was pre-warmed to either 30^0C or 37^0C. Incubations were continued with 300 rpm shaking for an additional 2 or 22 hours at either 30^0C or 37^0C, followed by RNA extraction and Northern blotting. RNA from equal numbers of cells was loaded in each lane. RNA isolation, agarose gel electrophoresis, and transfer were performed as described (Sherman, *et al.*, 1986; Sambrook, *et al.*, 1989; Davis, *et al.*, 1992). DNA fragments of *CYB2* (Guiard, 1985; primers CYB101 (5′ CGC TAA TAC AGT TCC CGC ATA 3′; positions -47 to -27 of the protein coding region) and CYB102 (5′ GGC TAT AAT CAT GCA TCC TCA 3′; positions +1,783 to 1,763); *CYC1* (Smith, *et al.*, 1979; primers CYC101, 5′ GGC ATG CAT GTG CTC TGT ATG 3′; positions -144 to -124); and CYC102, 5′ GGC GTG AAT GTA AGC GTG ACA 3′; positions +371 to +391) and *ACT1* (Gallwitz and Sures, 1980; primers ACT101 (5′ AAG GGG AAA GGG GGA TCC CAA TGG ATT CTG GTA TGT 3′; positions -2 to +16) and ACT102, 5′ AAA AAA GGA GGG GTC GAC GAT TAG AAA CAC TTG TGG 3′; positions +1,436 to +1,419) were produced by PCR (20 cycles;

temperature steps 94^{0}C, 1.5 min.; 50^{0}C, 3.0 min.; 72^{0}C, 2.5 min.), gel purified, and cloned into plasmid pUC19 by standard methods (Sambrook, *et al.*, 1989). DNA inserts were excised from plasmids with restriction endonucleases, gel-purified, and ^{32}P-labeled by random priming (Sambrook, *et al.*, 1989). Labeled probes were purified over Sephadex G-50 columns, and hybridized with filters in 5X SSC, 0.1% SDS, 5X Denhardt's solution, and 100 μg ml^{-1} sonicated, denatured salmon sperm DNA, either with 50% (v/v) formamide at 42^{0}C, or without formamide at 65^{0}C. Washing was performed as described (Davis, *et al.*, 1992). Blots were autoradiographed by exposure to Kodak XAR-5 film. Radioactivity on filters was measured using a Betagen counter. In reprobing experiments, blots were stripped by incubation in boiling water, and rehybridized separately with the indicated probe.

Disruption of CYB2.

Strains ED117-1 and ED119-1 were constructed by one-step disruption of *CYB2* in MB11 and MB11-3, respectively. A 2,264 base pair *CYB2* fragment was synthesized by polymerase chain reaction (PCR), as follows: MB11 DNA (*ca.* 10ng) was mixed in 100 μl reaction volumes containing 200 μM each dCTP, dGTP, dATP, and dTTP; 1 μM oligonucleotide primers CYB103 (5′ GGG TTG GGG AAG GAA TTC TCA CGC ATA CAT CGG AAG G 3′; positions -440 to -422) and CYB104 (5′ AAA AAA GGA GGG AAG CTT GCT AGG CTA TAA TCA TGC ATC CTC 3′; positions +1,788 to +1,765); and 5.0 units *Thermus aquaticus* DNA polymerase (Boehringer Mannheim). 30 cycles were performed, using

temperature steps of 94^{0}C, 1.5 min.; 50^{0}C, 3.0 min.; and 72^{0}C, 2.5 min. The DNA was digested with restriction endonucleases EcoRI and HindIII, gel purified, and ligated to EcoRI/HindIII-digested pUC19, to create pESD21. A 0.85kb ClaI fragment of the *S. cerevisiae TRP1* gene (Tschumper and Carbon, 1980; position -102 to +710 of the protein coding region) was inserted at the Cla I site (position +450) of *CYB2* in pESD21, to create pESD22, and a 1.1 kb Xba I fragment containing the *S. cerevisiae URA3* gene (Rose, *et al.*, 1984; position -227 to +943) was inserted into the Xba I site (position +593) of *CYB2*, to create pESD23. To generate substrates suitable for recombination at the *CYB2* locus, pESD22 and pESD23 were digested with EcoRI and PstI. Digested plasmid DNA was introduced into MB11 (Table 1) by electroporation as described (Becker and Guarente, 1991). Yeast genetic manipulations were performed as described (Sherman, *et al.*, 1986).

Results

CsA binding activity in cyclophilin disruption mutants

To define more specifically the nature of the *cpr3::HIS3* phenotype, we asked three questions: Is Cpr3 a *bona fide* cyclophilin, that is, does it bind CsA? Where is Cpr3 located in the cell? And, is the phenotype of *cpr3::HIS3* mutants caused by a defect in the induction of cytochrome genes in the nucleus, or in mitochondrial function?

The CsA-binding activities in yeast strains harboring all possible combinations of *CPR1*, *CPR2*, and *CPR3* disruption mutations were measured. Whole-cell extracts from MB11, and the seven yeast cyclophilin disruption mutant strains shown in Table 1 were prepared as described in Experimental Procedures. These extracts were then analyzed for their ability to bind ^{3}H-CsA using a Sephadex LH-20 column assay (Handschumacher, *et al.*, 1984). The calculated CsA-binding activities of each of these strains are presented in Table 2A.

Only disruption of *CPR1* leads to a consistently significant (50-70 percent) loss of soluble CsA-binding activity detectable by this assay (compare MB11 with MB12, and ED19 with ED21). No reproducibly significant loss of soluble CsA-binding activity upon disruption of either *CPR2* (compare MB11 with ED19, and MB12 with ED21) or *CPR3* (compare MB11 with MB11-3, and ED19 with ED20) is observed. There are several possible explanations. First, these proteins may be expressed below the detection limit of this assay. Second, the *CPR3* gene product may be membrane bound, and thus inaccessible to our extraction method (whereas

the *CPR2* gene product has been reported to be secreted; Zydowsky, *et al.*, 1992). Finally, the product of the *CPR4* gene (Frigerio and Pelham, 1993), or another, as yet undiscovered, yeast cyclophilin isoform might be contributing to a high residual background level of CsA-binding activity.

Specificity of Cpr3 antiserum

In order to assay Cpr3 expression in *E. coli* and to determine the cellular location of Cpr3, we raised two rabbit antisera directed against two distinct synthetic peptides of Cpr3: cpr3-1 and cpr3-2 (see Experimental Procedures). These peptide sequences are not found in any other known yeast cyclophilin protein. When used as probes in Western blots of *cpr1*, *cpr2*, and *cpr3* single disruption mutants, only the *cpr3::HIS3* strain MB11-3 showed a loss of an *ca.* 18.5 kd protein (Figure 1, A and B) when probed with either antiserum. This result is in good agreement with the predicted molecular weight of Cpr3 (19.9 kd for full-length Cpr3, 17.2 kd for Cpr3 without its predicted leader sequence). Therefore, the antisera Abcpr3-1 and Abcpr3-2 are specific for Cpr3.

Cpr3 expressed in Escherichia coli *binds CsA*

Since we were unable to detect a consistent loss of CsA binding activity in *cpr3::HIS3* strains, we expressed two different versions of *CPR3* in the bacterium *Escherichia coli* from the bacteriophage λ *plac-ptac* promoter. *E. coli* itself has no significant CsA-binding activity (Koletsky, *et al.*, 1986). We made three constructs. One, *CPR3FL*, contains the full-length protein-coding region of *CPR3*, including

the presumptive mitochondrial signal sequence. In the other construct, *CPR3Δ*, the leader sequence was deleted by omitting nucleotides for amino acids 2-22. *CPR3Δ* was used because it was uncertain what effect the leader sequence would have on the expression of Cpr3 in *E. coli*. We also made a *CPR1*-expressing construct as a positive control.

Figure 2 shows a Western blot of proteins from extracts prepared from induced (+IPTG) and uninduced bacteria containing each construct. Probing with antiserum Abcpr3-1 revealed the presence of an *ca.* 18 kd band in induced strains containing each of the *CPR3FL* constructs (ED102-1, ED102-2, and ED102-3), and the *CPR3Δ* constructs (ED103-1, ED103-2, and ED103-3). Cpr3 was not detected, as expected, in strains containing parent vector without insert (ED100) or with the *CPR1* construct (ED101).

We tested protein extracts from uninduced and induced strains for CsA binding. Table 2B shows the CsA binding activities calculated for each of the *E. coli* extracts. No CsA binding activity was detected in extracts from uninduced cultures (not shown). As expected, *E. coli* strain ED100 (pNH18 + no insert) displayed no significant CsA binding activity. Strain ED102-1, harboring the *CPR3FL* construct, did not produce detectable CsA binding activity. Strain ED103-1 expressed CsA-binding activity, at a level *ca.* one-fourth that of the strain containing the *CPR1* construct (compare strain ED101 with ED103). These results do not show the relative CsA binding affinity of Cpr1 and Cpr3, because the CsA-binding activities are not normalized to the amounts of expressed protein. Nevertheless, these results demonstrate that Cpr3 expressed without its leader

sequence in *E. coli* is a CsA binding protein.

Subcellular localization of Cpr3

We noted previously that the predicted *CPR3* protein sequence possesses an N-terminal leader sequence typical of those found in proteins targeted to mitochondria (Davis, et. al, 1992). In view of the phenotype of *cpr3*$^-$ strains, it was important to determine the subcellular localization of Cpr3. Accordingly, we isolated intact mitochondria from a wild-type yeast strain (MB11) by differential centrifugation (see Experimental Procedures). Mitochondria were further fractionated into intermembrane space, matrix, and a fraction containing both the inner and outer membranes. The purity of each subfraction was determined by assays for marker enzyme activity.

Figure 3A presents the results of marker enzyme analysis of the different subfractions. The intermembrane space and matrix fractions contain a minor (*ca.* 10-15%) degree of cross-contamination, as determined by flavocytochrome b_2 (intermembrane space marker) and fumarase (matrix marker) activity. The membrane fraction also contains *ca.* 15% contamination with fumarase activity. The peak of Cpr3 protein as determined by immunoblotting coincides with the peak of fumarase activity, the matrix marker enzyme (Figure 3B). These results demonstrate that Cpr3 is a component of the mitochondrial matrix.

Effect of CPR3 *inactivation on gene expression*

Growth on non-fermentable carbon sources requires induction of

cytochrome and other gene expression in the nucleus, as well as their function in mitochondria. Proper induction of cytochrome gene expression is dependent on a signal transduction pathway originating in the mitochondria (reviewed in Forsburg and Guarente, 1989; Lodi and Guarente, 1991). In view of the data that suggest that cyclophilins are involved in signal transduction, we wished to determine whether *cpr3::HIS3* yeast strains were capable of normal induction and maintenance of cytochrome gene expression. Strains MB11 (*CPR3*) and MB11-3 (*cpr3::HIS3*) were initially grown under repressing conditions (glucose) at 30^0C; glucose represses the transcription of *CYB2* and *CYC1* (Guiard, 1985; Forsburg and Guarente, 1989). The cultures were then shifted to rich medium supplemented with lactate to induce cytochrome gene transcription. Inductions were continued at either 30^0C or 37^0C for 2 or 22 hours. All samples were then analyzed for *CYC1* (gene for iso-1-cytochrome c; Sherman, *et al.*, 1966), *CYB2* (gene for flavocytochrome b_2; Guiard, *et al.*, 1985), and *ACT1* (gene for actin; Gallwitz and Sures, 1980) mRNA accumulation by Northern hybridization.

Figure 4A presents the results of a Northern blot from this experiment. Figure 4B shows the amount of *CYB2* and *CYC1* mRNA normalized to *ACT1* mRNA levels. These data show that cytochrome gene induction is not significantly reduced at 30^0C for 2 or 22 hours as a consequence of *CPR3* inactivation. This finding is significant in view of our observation that, at 30^0C, the average generation time of strain MB11-3 is reproducibly more than 50 percent greater than that of strain MB11 (Table 3). Thus, strain MB11-3 (*cpr3::HIS3*) grows more slowly than MB11 (*CPR3*), although induction of

cytochrome genes is not significantly compromised. Therefore, the growth defect of *cpr3::HIS3* mutants is not due to a defect in the signal transduction events leading from the mitochondria to the nucleus.

After 2 hours at 37^{0}C, both strains display decreased accumulation of *CYB2* and *CYC1* mRNA; this decrease is greater in strain MB11-3. However, by 22 hours at 37^{0}C, there is no difference, normalized to *ACT1*, in the relative accumulation of *CYB2* mRNA between mutant and wild-type. We see a similar situation when analyzing the induction of *CYC1* in *cpr3::HIS3* strains. At 37^{0}C, there is a delay in the induction of *CYC1* mRNA in the *cpr3::HIS3* strain, but by 22 hours the level of *CYC1* mRNA normalized to *ACT1* mRNA in the *cpr3::HIS3* mutant is somewhat greater than that of strain MB11-3.

We observe a difference in the amount of mRNA per cell between strains MB11 and MB11-3 at 37^{0}C, as inferred by the levels of the constitutively expressed *ACT1* mRNA. We attribute this difference in mRNA per cell to the metabolic state of the strains, reflected in the generation times observed under these conditions (Table 4); i.e., a large increase in the generation time corresponds to a decrease in the amount of mRNA per cell. Interpretation of the results at 37^{0}C is problematic, due to the poor metabolic state of the cells. However, the clear result at 30^{0}C is that *cpr3::HIS3* strains are not significantly hampered in their ability to induce cytochrome gene transcription, but are nonetheless affected in mitochondrial function.

We conclude from our findings that Cpr3 is not required for the signal transduction pathway that governs cytochrome gene expression.

Effect of CPR3 *inactivation on flavocytochrome b_2 activity*

We explored the possibility that Cpr3 could be essential for the folding of flavocytochrome b_2, a lactate dehydrogenase located in the intermembrane space of mitochondria (Daum and Schatz, 1982) and reported to be essential for growth on lactate medium (Guiard, 1985). We incubated yeast strains MB11 and MB11-3 in glycerol medium at 30^0C and 37^0C, for 22 hours, and then assayed flavocytochrome b_2 activity. Figure 5 shows that inactivation of *CPR3* leads to *ca.* a 22% decrease at in flavocytochrome b_2 activity 30^0C, and an *ca.* 90% reduction at 37^0C. Again, interpreting the results at 37^0C is problematic because of the poor growth state of the cells. The slight decrease at 30^0C seems insufficient to cause the increase in average generation time observed in *cpr3::HIS3* strains (Table 3).

To test more directly whether the phenotype of *cpr3::HIS3* strains is caused by a deficiency in flavocytochrome b_2, we constructed yeast strains with two different insertion mutations of the *CYB2* gene. The constructs used for disruption are shown in Figure 6A. These mutants do not express appropriately sized *CYB2* mRNA (not shown), and have no detectable flavocytochrome b_2 activity (Figure 6B). However, these mutants are able to grow on lactate medium at both 30^0C and 37^0C (Figure 6c). These strains also grow on both rich medium lacking any added carbon source, and synthetic medium supplemented only with amino acids, indicating that they are metabolizing carbon sources other than lactic acid (probably amino acids; result not shown). In addition, the *cpr3::HIS3* phenotype on rich medium lacking added carbon source is similar to that observed for growth on lactate medium (result not shown). Two conclusions can be drawn

from these results. First, inhibition of flavocytochrome b_2 is not sufficient to account for the observed phenotype of *cpr3::HIS3* mutants. Second, the function of Cpr3 is not limited to lactate utilization. Instead, Cpr3 plays a general role in mitochondrial function.

Discussion

Previously, we demonstrated that the *S. cerevisiae CPR3* gene is essential for growth on lactate medium at 37^{0}C (Davis, *et al.*, 1992). In this paper, we have defined more specifically the nature of the *CPR3* gene product, Cpr3, and the physiological defect in *cpr3::HIS3* mutant strains. First, Cpr3 expressed in bacteria binds CsA, a finding consistent with those of McLaughlin, *et al.* (1992), who isolated Cpr3 as a CsA-sensitive PPIase. More significantly, we have localized Cpr3 in the mitochondrial matrix. This result limits the explanation of the phenotype of *cpr3::HIS3* mutants, and restricts the analysis of the biochemistry of Cpr3 to mitochondrial processes.

Based on our previous finding that Cpr3 is essential for growth on lactate at 37^{0}C, and in view of the evidence that cyclophilins may be involved in signal transduction, we tested the following possibilities. First, Cpr3 could be necessary for signal transduction events (which might or might not require protein folding), leading from mitochondria to the expression of cytochrome genes in the nucleus. Second, the phenotype of *cpr3::HIS3* mutants could be caused by a deficiency in the proper folding of proteins involved in mitochondrial metabolism, such as flavocytochrome b_2, a yeast lactate dehydrogenase reported to be essential for growth on lactate medium (Guiard, *et al.*, 1985).

Earlier, we had determined that *CPR3* is not essential for *CYB2* expression during 2 hours at 37^{0}C (Davis, *et al.*, 1992). Here, we further probe the effect of *CPR3* inactivation on cytochrome gene expression. We observe no reduction in

CYB2 and *CYC1* mRNA levels resulting from a *cpr3::HIS3* mutation at 30^{0}C, despite a more than 50 percent increase in average generation time relative to wild-type. This finding demonstrates that Cpr3 is not essential for the signal transduction events controlling the expression of these genes.

Flavocytochrome b_2 activity is only slightly reduced in a *cpr3::HIS3* mutant at 30^{0}C, while growth is more severely affected. At 37^{0}C, flavocytochrome b_2 activity is severely impaired as a consequence of the *cpr3::HIS3* mutation, but the interpretation of these data is complicated by the poor growth state of the cells. However, strains harboring null mutations of *CYB2* are able to grow under conditions that prohibit the growth of *cpr3::HIS3* mutants. Therefore, inhibition of flavocytochrome b_2 activity is insufficient to explain the phenotype of *cpr3::HIS3* mutants.

It is clear from these results that the relevant biochemical target(s) of Cpr3 are proteins other than flavocytochrome b_2. Based on these findings, and the knowledge that growth of *cpr3::HIS3* mutants is hampered on other non-fermentable carbon sources (Davis, *et al.*, 1992), we conclude that Cpr3 plays a general role in mitochondrial function at all temperatures, one not limited to growth on lactic acid. To define and characterize this role more clearly, it is important to identify biochemical targets of the *CPR3* gene product. We have isolated strains harboring extragenic suppressors of *cpr3::HIS3* (E. S. D. and M. B. B., manuscript in preparation), which might help facilitate the realization of this goal.

References

Becker, D. M. and Guarente, L. (1991). *Meth. Enzymol.* **194**, 182-187.

Borel, J. F., Feurer, C., Gubler, H. U., & Staehlein, H. (1976). *Agents and Actions* **6**, 468-475.

Borel, J. F., Feurer, C., Magnee, C. & Staehlein, H. (1977). *Immunology* **32**, 1017-1025.

Bradford, M. (1976). *Anal. Biochem.* **72**, 248-254.

Clipstone, N. A. and Crabtree, G. R. (1992). *Nature* **357**, 695-697.

Colley, N. J., Baker, E. K., Stamnes, M. A., & Zuker, C. S. (1991). *Cell* **67**, 255-263.

Daum, G., Böhni, P. C., & Schatz, G. (1982). *J. Biol. Chem.* **257**, 13028-13033.

Davis, E. S., Becker, A., Heitman, J., Hall, M. N., & Brennan, M. B. (1992). *Proc. Natl. Acad. Sci. U. S. A.* **89**, 11169-11173.

Emmel, E. A., Verweij, C. L., Durand, D. B., Higgins, K. M., Lacy, E., &

Crabtree, G. R. (1989). *Science* **246**, 1617-1620.

Flanagan, W. M., Corthesy, B., Bram, R. J., & Crabtree, G. R. (1991). *Nature* **352**, 803-807.

Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., & Schmid, F. X. (1989) *Nature* **337**, 476-478.

Forsburg, S. L. & Guarente, L. (1989). *Ann. Rev. Cell Biol.* **5**, 153-180.

Frigerio, G. & Pelham, H. R. B. (1993). *J. Mol. Biol.* **233**, 183-188.

Gallwitz, D. & Sures, I. (1980). *Proc. Natl. Acad. Sci. U. S. A.* **77**, 2546-2550.

Guiard, B. (1985). *EMBO J.* **4**, 3265-3272.

Handschumacher, R. E., Harding, M. W., Rice, J., Drugge, R. J., & Speicher, D. W. (1984). *Science* **226**, 544-547.

Harlow, E. & Lane, D. (1988). *Antibodies: A laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Hasan, N. & Szybalski, W. (1987). *Gene* **56**, 145-151.

Koletsky, A. J., Harding, M. W., & Handschumacher, R. E. (1986). *J. Immunol.* **137**, 1054-1059.

Krönke, M. Leonard, W. J., Depper, J. M., Arya, S. K., Wong-Staal, F., Gallo, R. C., Waldmann, T. A., & Greene, W. C. (1984). *Proc. Natl. Acad. Sci. U. S. A.* **81**, 5214-5218.

Labeyrie, F., Baudras, A., & Lederer, F. (1978). *Meth. Enzymol.* **53**, 238-256.

Laemmli, U. K. (1970). *Nature* **227**, 680-685.

Liu, J., Farmer, Jr., J. D., Lane, W. S., Friedman, J., Weissman, I., & Schreiber, S. L. (1991) *Cell* **66**, 807-815.

Lodi, T. & Guiard, B. (1991). *Mol. Cell. Biol.* **11**, 3762-3772.

McLaughlin, M. M., Bossard, M. J., Koser, P. L., Cafferkey, R., Morris, R. A., Miles, L. M., Strickler, J., Bergsma, D. J., Levy, M. A., & Livi, G. P. (1992). *Gene* **111**, 85-92.

O' Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J., & O'Neill, E. A. (1992). *Nature* **357**, 692-694.

Racker, E. (1950). *Biochem. Biophys. Acta* **4**, 211-214.

Rose, M., Grisafi, P., & Botstein, D. (1984). *Gene* **29**, 113-124.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., & Arnheim, N. (1985). *Science* **230**, 1350-1354.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sherman, F., Stewart, J. W., Margoliash, E., Parker, J., & Campbell, W. (1966). *Proc. Natl. Acad. Sci. U. S. A.* **55**, 1498-1504.

Sherman, F., Fink, G. R., & Hicks, J. B. (1986). *Laboratory course manual for methods in yeast genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Shieh, B-H., Stamnes, M. A., Seavello, S., Harris, G. L., & Zuker, C. S. (1989) *Nature* **338**, 67-70.

Sigal, N. H., Dumont, F., Durette, P., Siekierka, J. J., Peterson, L., Rich, D. H., Dunlap, B. E., Staruch, M. J., Melino, M. R., Koprak, S. L., Williams, D., Witzel, B., & Pisano, J. M. (1991). *J. Exp. Med.* **173**, 619-628.

Smith, M., Leung, D. W., Gillam, S., Astell, C. R., Montgomery, D., & Hall, B. D. (1979). *Cell* **16**, 753-761.

Takahashi, N., Hayano, T., & Suzuki, M. (1989) *Nature* **337**, 473-4751.

Tropschug, M., Barthelemess, I. B., & Neupert, W. (1989) *Nature* **342**, 953-955.

Tschumper, G. & Carbon, J. (1980). *Gene* **10**, 157-166.

Zydowsky, L. D., Ho, S. I., Baker, C. H., McIntyre, K., & Walsh, C. T. (1992). *Protein Sci.* **8**, 961-969.

Table 1. Yeast Strains.

Strain	Genotype
MB11	*MATα ade2-101 trp1-Δ1 ura3-52 his3-Δ200 lys2-801 can1*
MB11-3	MB11 *cpr3::HIS3*
MB12	MB11 *cpr1::URA3*
MB12-1	MB11 *cpr1::URA3 cpr3::HIS3*
ED19	MB11 *cpr2::TRP1*
ED20	MB11 *cpr2::TRP1 cpr3::HIS3*
ED21	MB11 *cpr1::URA3 cpr2::TRP1*
ED22	MB11 *cpr1::URA3 cpr2::TRP1 cpr3::HIS3*
ED117-1	MB11 *cyb2::TRP1*
ED119-1	MB11 *cyb2::URA3*

Table 2. CsA-binding measurements

A. Yeast extracts

Strain	**pmol CsA bound per mg protein**
MB11	91.0 ± 13.1 (n = 5)
MB11-3 (MB11 *cpr3*)	86.0 ± 22.0 (n = 5)
MB12 (MB11 *cpr1*)	42.0 ± 9.9 (n = 5)
MB12-1 (MB11 *cpr1 cpr3*)	28.0 ± 12.6 (n = 5)
ED19 (MB11 *cpr2*)	79.0 ± 19.8 (n = 2)
ED20 (MB11 *cpr2 cpr3*)	61.0 ± 39.6 (n = 2)
ED21 (MB11 *cpr1 cpr2*)	35.5 ± 9.2 (n = 2)
ED22 (MB11 *cpr1 cpr2 cpr3*)	22.5 ± 13.4 (n = 2)

B. Bacterial extracts

Strain	**pmol CsA bound per mg protein**
ED101 (pNH18, no insert)	0 (n = 2)
ED102 (pNH18 + *CPR1*)	12.3 ± 3.9 (n = 2)
ED103 (pNH18 + *CPR3FL*)	0 (n = 2)
ED104 (pNH18 + *CPR3Δ*)	3.7 ± 0.7 (n = 2)

In A and B, values indicate the average of the indicated number of trials

Table 2. (continued)

(n), plus or minus the standard error measurement. Protein extracts (55-400 μg for yeast extracts, 200 μg for bacterial extracts) were mixed in glass tubes or siliconized microcentrifuge tubes with 7.5 μl fetal bovine serum and 10 μl 50 μM CsA (containing a mixture of unlabeled and ^{3}H-labeled CsA) in 100 μl volumes. Reactions were gently rocked on a Nutator at room temperature for 20 minutes. Reactions were then loaded on 1.8 ml LH-20 chromatography columns (BioRad PolyPrep) equilibrated in binding buffer (0.02M Tris-HCl, pH 7.5). Binding buffer was applied to the column, and four 0.5ml fractions were collected in glass scintillation vials containing liquid scintillation cocktail (Beckman Ready-Safe). Two peaks of radioactivity were observed, in fractions 2 (void volume) and 4. CsA binding activity was determined by calculating the fraction of the total radioactivity in the reaction that was collected in fraction 2 (void volume).

Table 3. Average generation times during induction on lactate medium.

Strain	temperature (^{0}C)	Average generation time (hours)
MB11 (*CPR3*)	30	4.3
	37	16.1
MB11-3 (*cpr3::HIS3*)	30	7.1
	37	51.0

The cultures were those used for the Northern blotting analysis of Figure 4. Average generation times were computed from the difference in optical density measurements at 600nm taken 2 and 22 hours after inoculation into induction (lactate) medium.

Figure 1. Specificity of Cpr3 peptide-specific antisera.

50 μg of total protein extracts of strains MB11 (*CPR1*, *CPR2*, *CPR3*), MB11-3 (*CPR1*, *CPR2*, *cpr3::HIS3*), MB12 (*cpr1::URA3*, *CPR2*, *CPR3*) and ED19 (*CPR1*, *cpr2::TRP1*, *CPR3*) were electrophoresed on discontinuous SDS-polyacrylamide gels (4% stacking gel, 15% separating gel; 7.0 cm X 8.0 cm X 0.075 cm) at 200 volts for 45 min. Gels were transferred electrophoretically to PVDF membranes by standard methods (Harlow and Lane, 1988). Immunoblots were probed with Cpr3 peptide-specific antisera: A, Abcpr3-1; or B, Abcpr3-2 (see Experimental Procedures). Beneath each blot is a duplicate gel, Coomasie blue stained for total protein. To the left of each blot are molecular weight standards, in kilodaltons, determined from electrophoresis of a prestained mixture of standards (BioRad, low-range markers).

Figure 1.

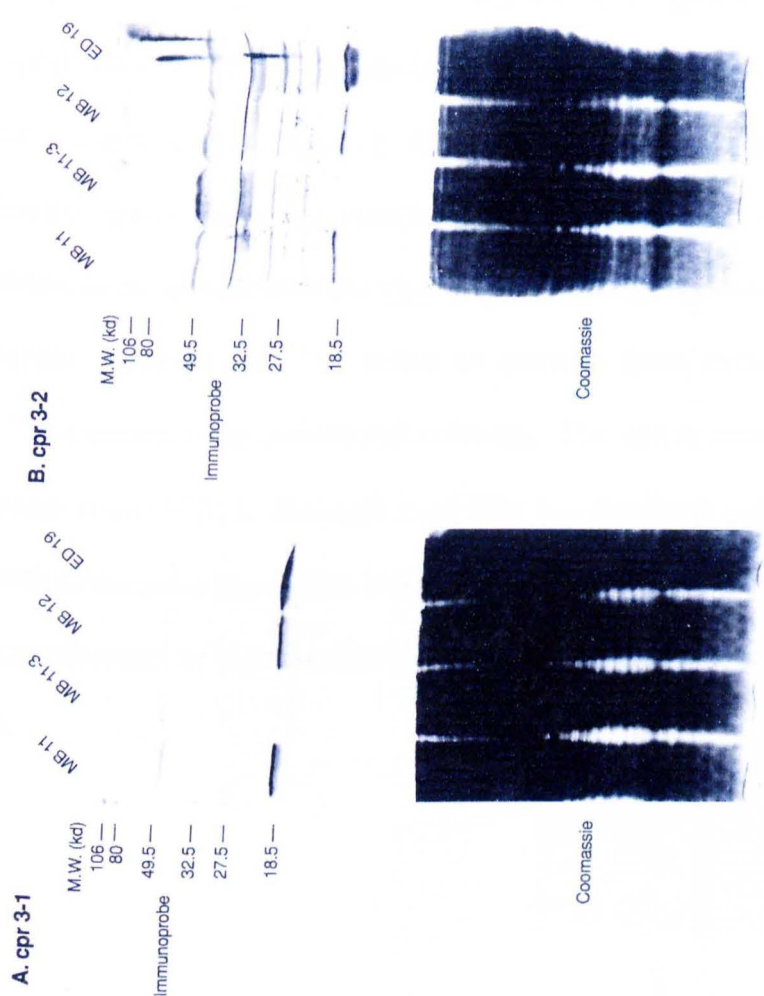

A. cpr 3-1
M.W. (kd)
106
80
49.5
32.5
27.5
18.5
Immunoprobe
MB 11
MB 11-3
MB 12
ED 19
Coomassie
B. cpr 3-2
M.W. (kd)
106
80
49.5
32.5
27.5
18.5
Immunoprobe
MB 11
MB 11-3
MB 12
ED 19
Coomassie

Figure 2. Expression of *CPR3FL* and *CPR3Δ* in *E. coli*.

50 μg of protein extracts from yeast strain MB11 (*CPR3*) and bacterial strains ED100 (plasmid pNH18, no insert), ED102-1 (pNH18 + *CPR3FL*), ED102-2 (pNH18 + *CPR3FL*), ED102-3 (pNH18 + *CPR3FL*), ED103-1 (pNH18 + *CPR3Δ*), ED103-2 (pNH18 + *CPR3Δ*), ED103-3 (pNH18 + *CPR3Δ*), and ED101 (pNH18 + *CPR1*) were electrophoresed on SDS-polyacrylamide gels as described in the legend to Figure 1. Gels were transferred electrophoretically to nitrocellulose membranes by standard methods (Harlow and Lane, 1988). Immunoblots were probed with the Cpr3 peptide-specific antiserum Abcpr3-1 (see Experimental Procedures). "+" refers to extracts from cultures induced with IPTG; "-" to extracts from uninduced cultures. The arrow shows the position of Cpr3 in yeast strain MB11. Beneath each blot is a duplicate gel, stained for total protein with Coomassie blue. The molecular weight markers, in kilodaltons, were from electrophoresis of a prestained mixture of standards (BioRad, Low-Range Markers).

Figure 2.

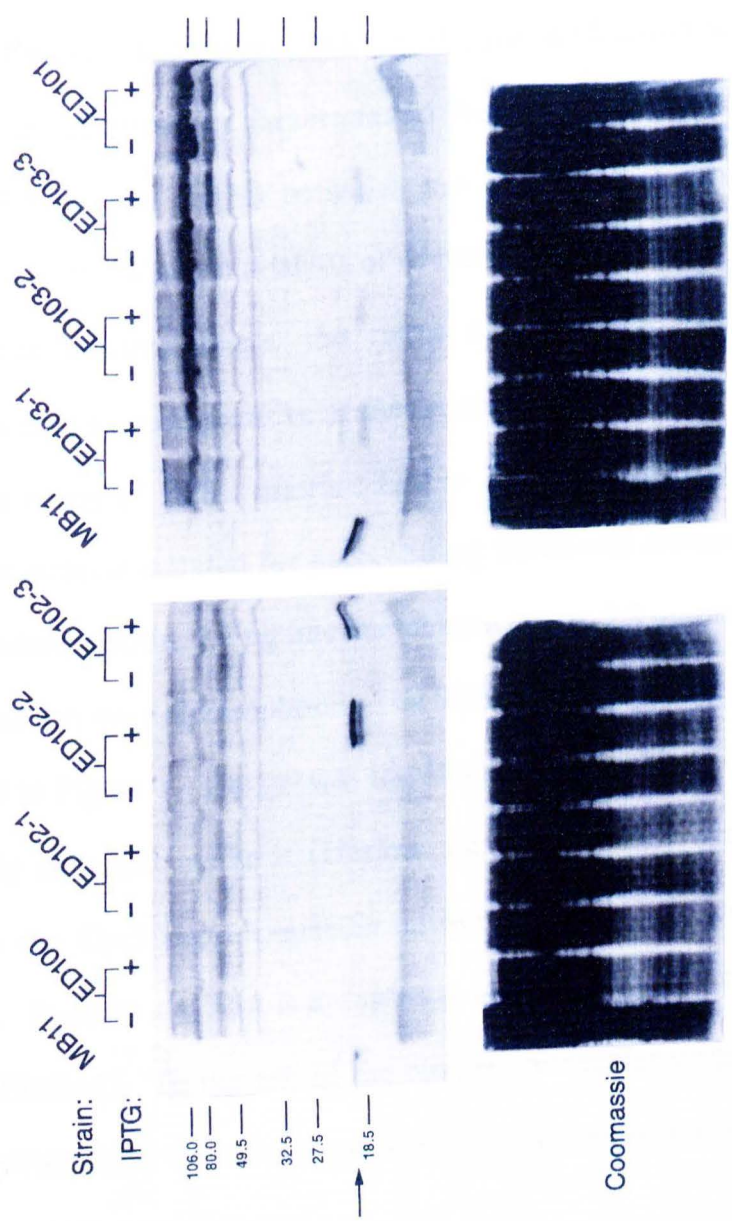

Strain:
IPTG:
MB11
ED100
ED102-1
ED102-2
ED102-3
MB11
ED103-1
ED103-2
ED103-3
ED101
106.0
80.0
49.5
32.5
27.5
18.5
Coomassie

Figure 3. Subcellular localization of Cpr3.

A. Marker enzyme activities of submitochondrial fractions. IMS, intermembrane space. Matrix, mitochondrial matrix space. OM + IM, inner and outer mitochondrial membranes. 4.0 μg of each subfraction was asssayed for marker enzyme activities (see Expermiental Procedures). Both flavocytochrome b_2 (left) and fumarase (right) activities are expressed as percentage of the calculated total. B. Western analysis of whole cell, cytosolic, and mitochondrial fractions. For electrophoresis, the ratios of whole-cell extract : cytosol : mitochondria are *ca.* representative of the ratios of total protein isolated for each. Likewise, the ratios of IMS : matrix : OM + IM are *ca.* representative of the ratios of total protein isolated for each. 50 μg whole-cell extract, 43 μg cytosol, 5 μg whole mitochondria, 1.1 μg intermembrane space, 2.2 μg matrix, and 1.7 μg IM + OM fraction were electrophoresed on SDS-polyacrylamide gels as described in the legend to Figure 1. The gel was transferred electrophoretically to a PVDF membrane by standard methods (Harlow and Lane, 1988). Immunoblot was probed with the Cpr3 peptide-specific antiserum Abcpr3-1 (see Experimental Procedures). Beneath the blot is a duplicate gel, silver-stained for total protein using a kit (BioRad). To the left of the blot are molecular weight standards, in kilodaltons, determined from electrophoresis of a prestained mixture of standards (BioRad, low-range markers).

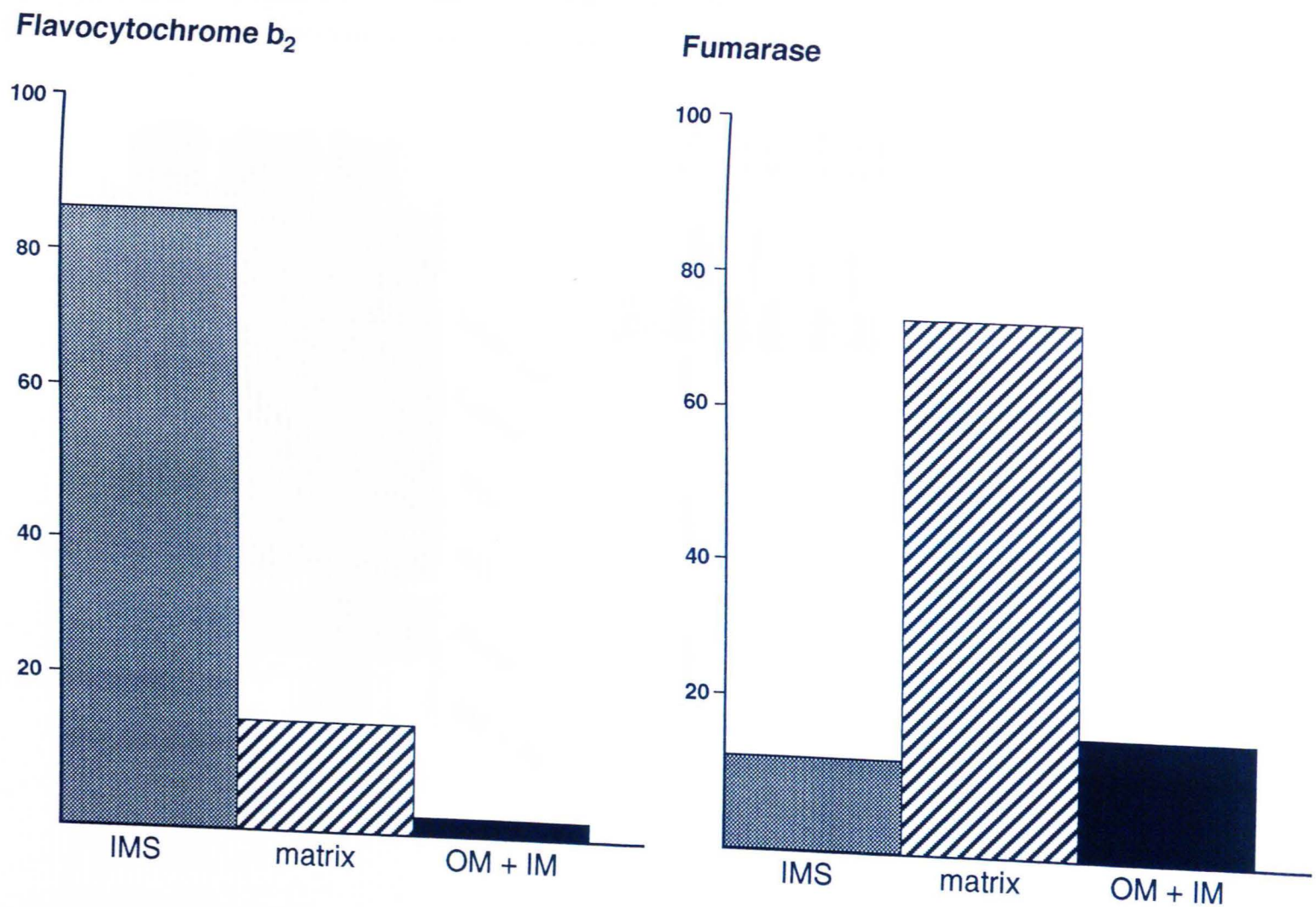

Flavocytochrome b_2
100
80
60
40
20
IMS
matrix
OM + IM
Fumarase
100
80
60
40
20
IMS
matrix
OM + IM

Figure 3A.

Figure 3B.

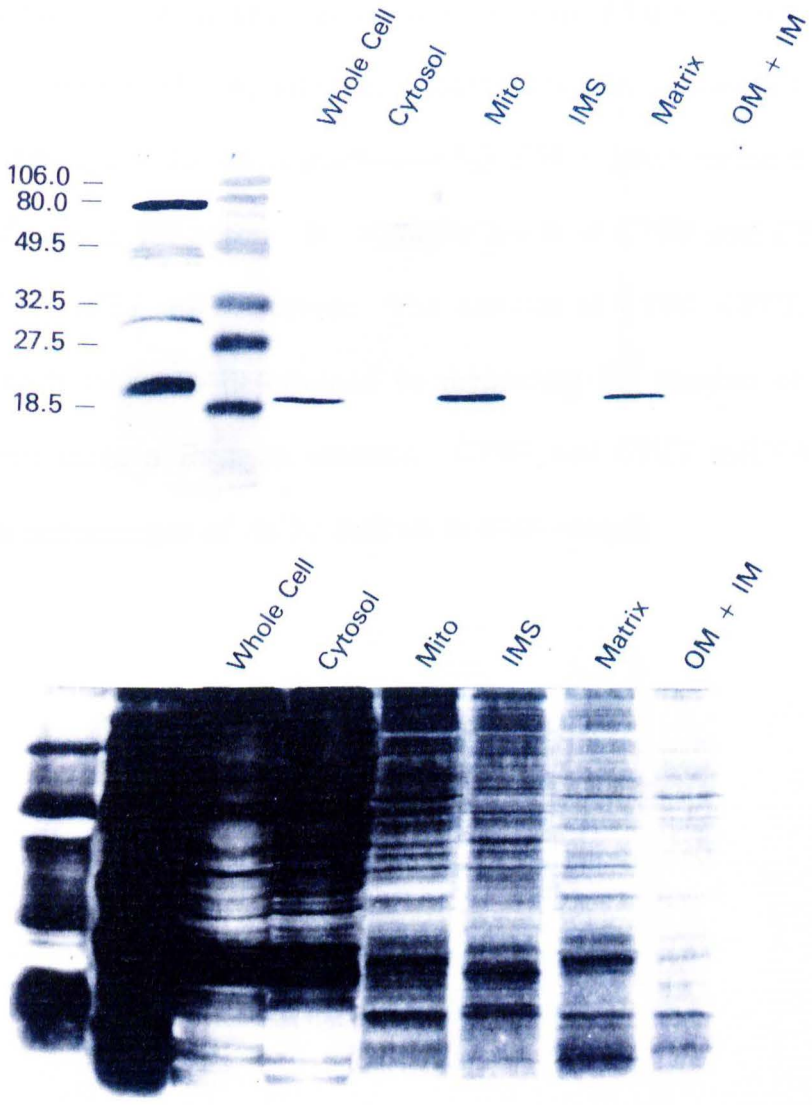

Whole Cell
Cytosol
Mito
IMS
Matrix
OM + IM
106.0
80.0
49.5
32.5
27.5
18.5
Whole Cell
Cytosol
Mito
IMS
Matrix
OM + IM

Figure 4. Analysis of cytochrome gene expression in a *cpr3::HIS3* mutant.

A. Northern blot analysis of mRNA from strains induced at 30^0C and 37^0C. RNAs extracted from strains MB11 (*CPR3*) and MB11-3 (*cpr3::HIS3*) incubated in 10.0% glucose (D) or in 2.0% D-, L-lactate (L) for 2 or 22 hours were electrophoresed in 1.0% agarose gels in the presence of 3.0% (w/v) formaldehyde, and transferred to a nylon membrane. Membranes were probed with ^{32}P-labeled DNA for *CYB2* (gene for flavocytochrome b_2), *CYC1* (gene for iso-1-cytochrome c) and *ACT1* (gene for actin). B. Relative levels of *CYB2* and *CYC1* mRNA, normalized to *ACT1* mRNA levels. The amount of *CYB2*, *CYC1*, and *ACT1* mRNA in each lane was determined by measuring the number of counts per minute (cpm) using a Betagen counter. *CYB2* and *CYC1* mRNA levels are expressed as percentages of *ACT1* mRNA in each sample.

Figure 4A.

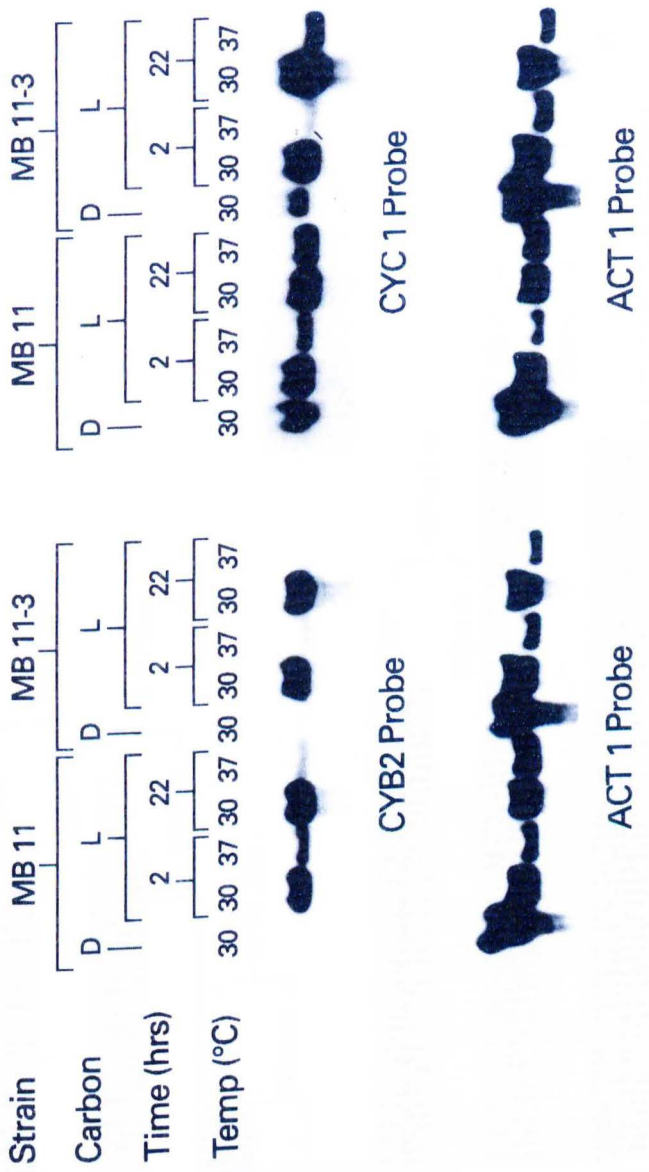

Strain
Carbon
Time (hrs)
Temp (°C)
MB 11
MB 11-3
D
L
2
22
30 30 37 30 37 30 30 37 30 37
CYB2 Probe
ACT 1 Probe
CYC 1 Probe
ACT 1 Probe

Figure 4B.

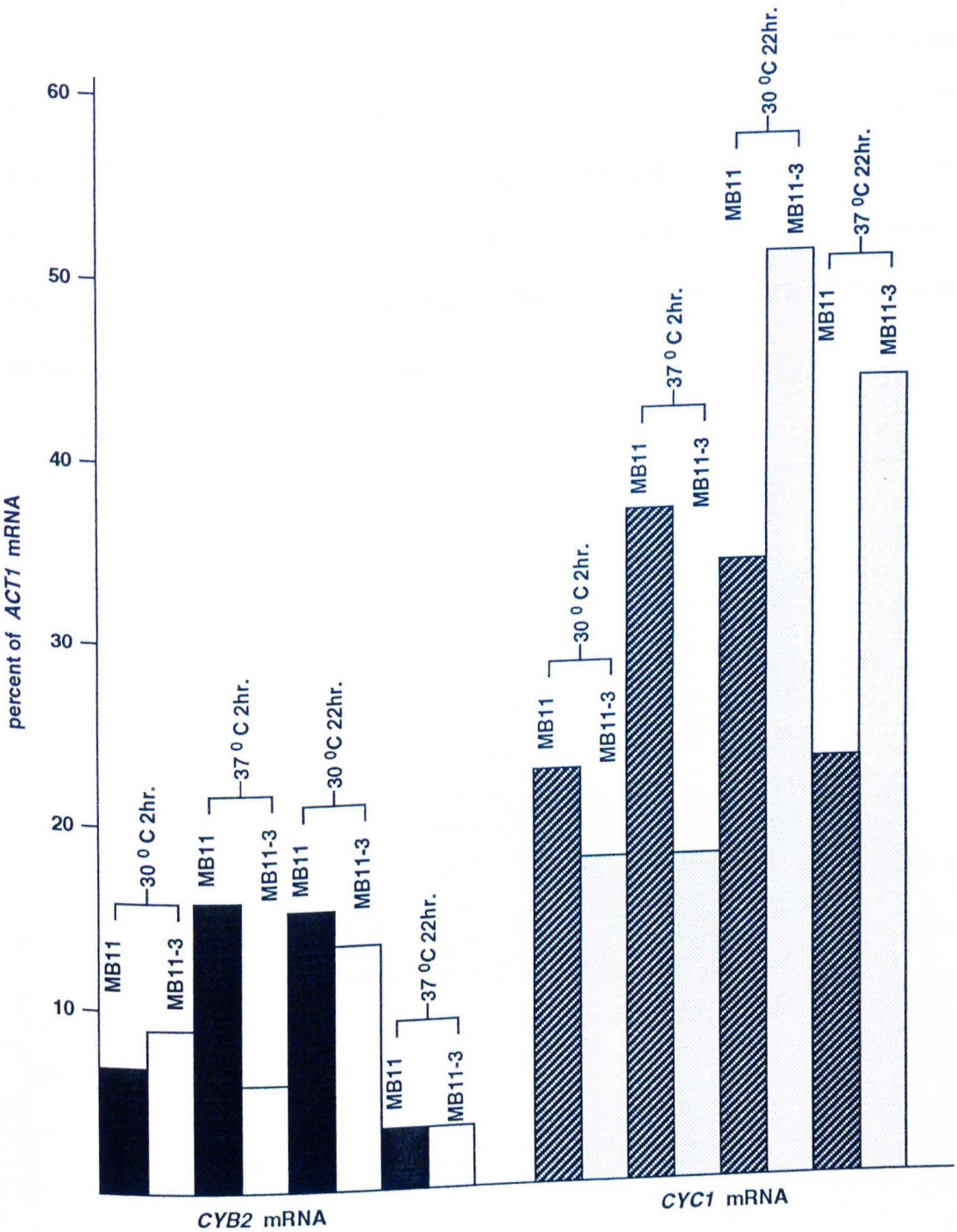

percent of ACT1 mRNA
60
50
40
30
20
10
MB11
30 °C 2hr.
MB11-3
MB11
37 °C 2hr.
MB11-3
MB11
30 °C 22hr.
MB11-3
MB11
37 °C 22hr.
MB11-3
CYB2 mRNA
MB11
30 °C 2hr.
MB11-3
MB11
37 °C 2hr.
MB11-3
MB11
30 °C 22hr.
MB11-3
MB11
37 °C 22hr.
MB11-3
CYC1 mRNA

Figure 5. Analysis of flavocytochrome b_2 activity in a *cpr3::HIS3* mutant.

Strains MB11 (*CPR3*; shaded bars) and MB11-3 (*cpr3::HIS3*; cross-hatched bars) were incubated in YP medium supplemented with glycerol at 30^0C and 37^0C for 22 hours. Whole-cell extracts (220 μg each sample) were assayed twice each for flavocytochrome b_2 activity (see Experimental Procedures). Activity is expressed as units per mg, multiplied by 100. The data are from a representative of three independent experiments.

Figure 5.

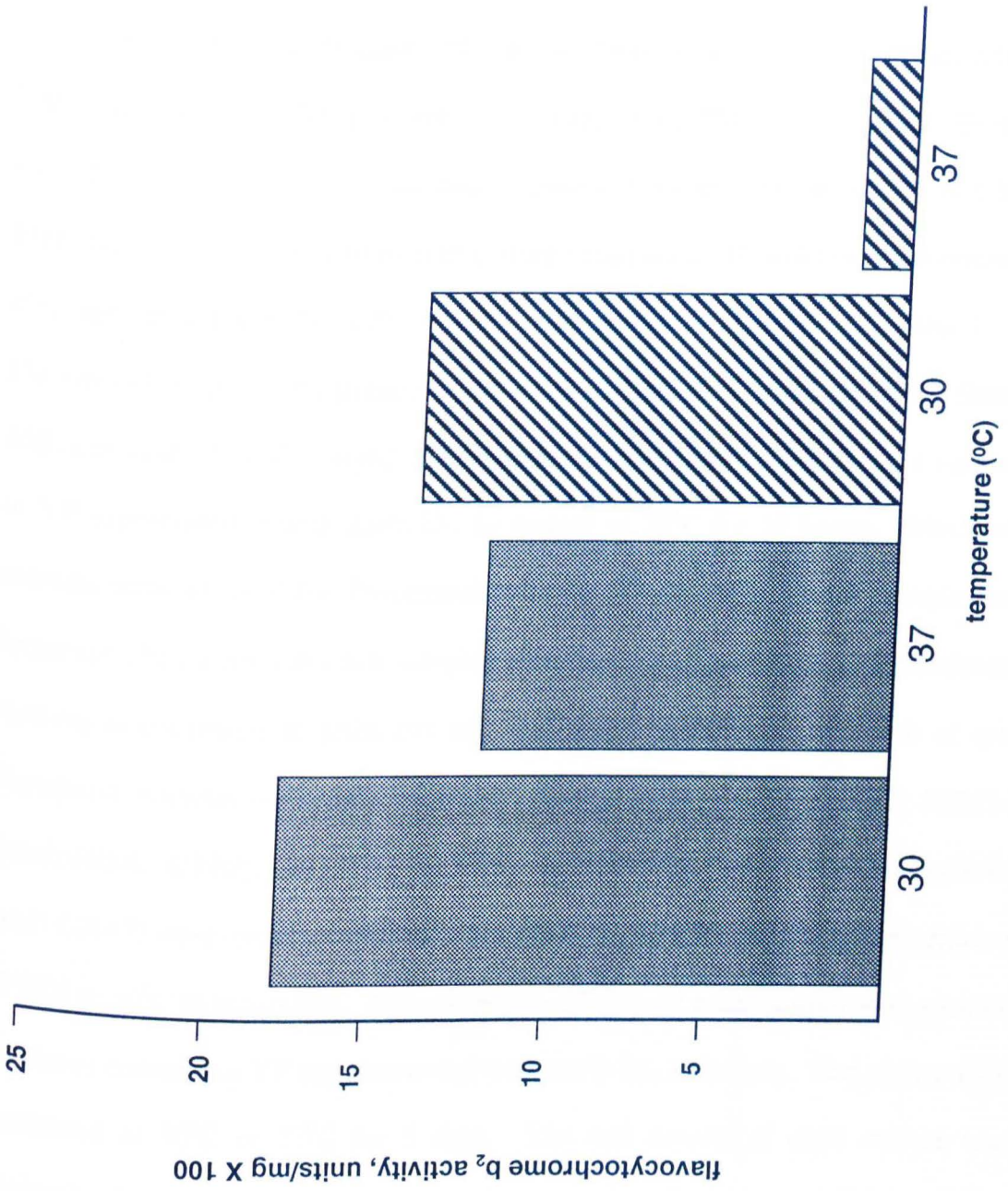

flavocytochrome b2 activity, units/mg X 100
25
20
15
10
5
30
37
30
37
temperature (°C)

Figure 6. Loss of flavocytochrome b_2 is insufficient to explain the phenotype of *cpr3::HIS3* mutants.

A. Schematic diagrams of the structures of the *CYB2* insertion alleles. Top, wild-type (*CYB2*$^+$) allele. Middle, *cyb2::TRP1* construct. Bottom, *cyb2::URA3* construct. Stippled boxes represent protein coding regions of *CYB2*. Flanking solid lines represent non-coding sequences. Restriction endonuclease cleavage sites are indicated: E, Eco RI; P, Pst I; C, Cla I; X, Xba I. B. Flavocytochrome b_2 and fumarase activities of *cyb2* disruption mutants. Strains MB11 (*CYB2*), ED117-1 (*cyb2::TRP1*) and ED119-1 (*cyb2::URA3*) were cultured in YP supplemented with 2.0% D-, L- lactate at 30^{0}C for 19 hours. Whole-cell extracts were assayed for flavocytochrome b_2 (77 μg protein each sample) and fumarase (10 μg protein each sample) activities (see Experimental Procedures). Activity is expressed as units per mg, multiplied by 100. C. Growth of *cyb2* disruption mutants on lactate medium. Strains MB11 (*CPR3*, *CYB2*) MB11-3 (*cpr3::HIS3*, *CYB2*), ED117-1 (*CPR3*, *cyb2::TRP1*), and ED119-1 (*CPR3*, *cyb2::URA3*) were initially cultured overnight in liquid YP containing 2.0% glucose (YPD) at 30^{0}C to saturation. Approximately 25 cells of each sample were spotted on plates containing YP supplemented with 2.0% D-, L-lactate. The plates were incubated at 30^{0}C or 37^{0}C for 5 days. The cell density of each culture was determined prior to spotting by plating dilutions on YPD plates at 30^{0}C for 2 days. Beneath the plates is a schematic diagram of the strain arrangement.

Figure 6A.

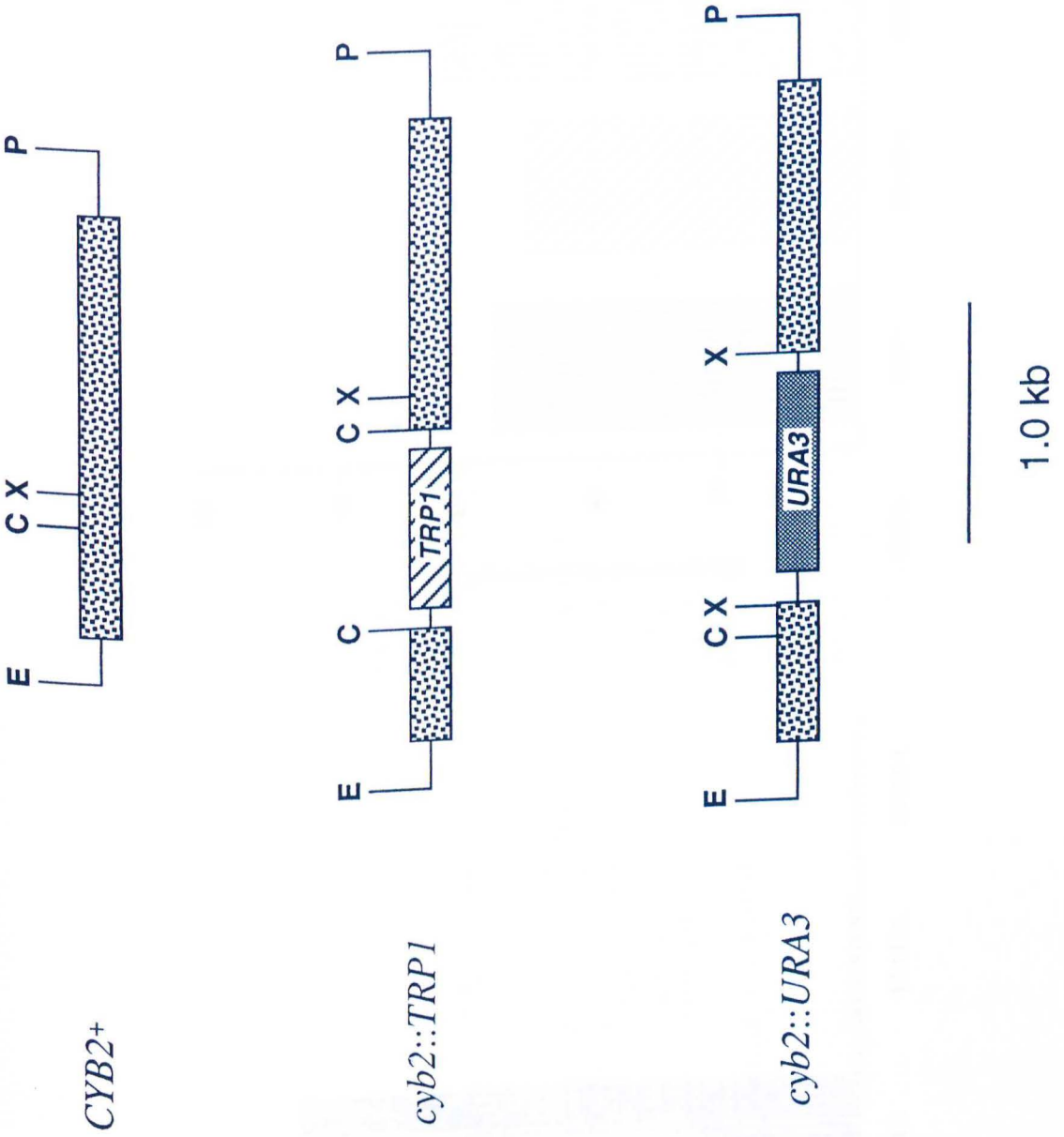


Figure 6B.

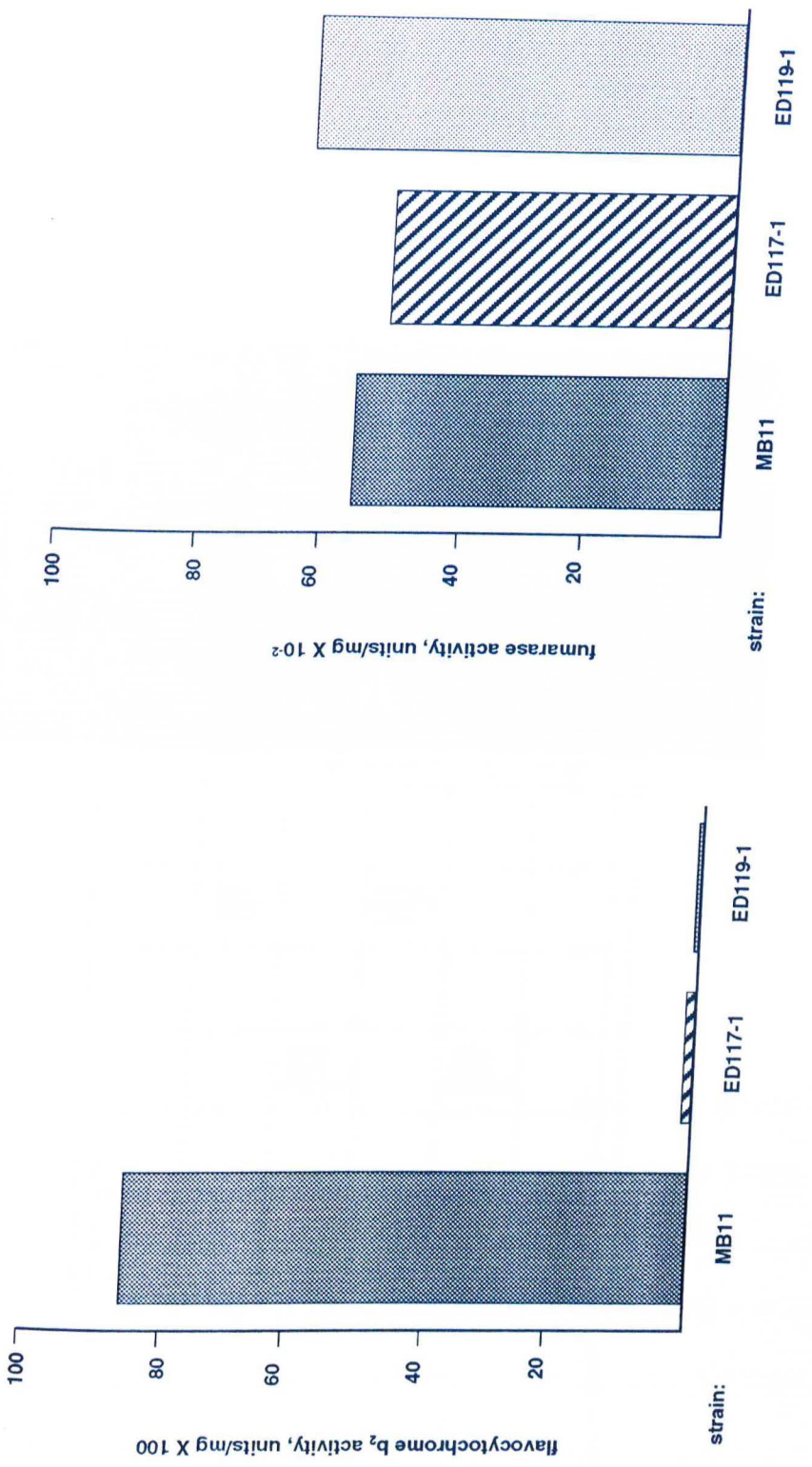
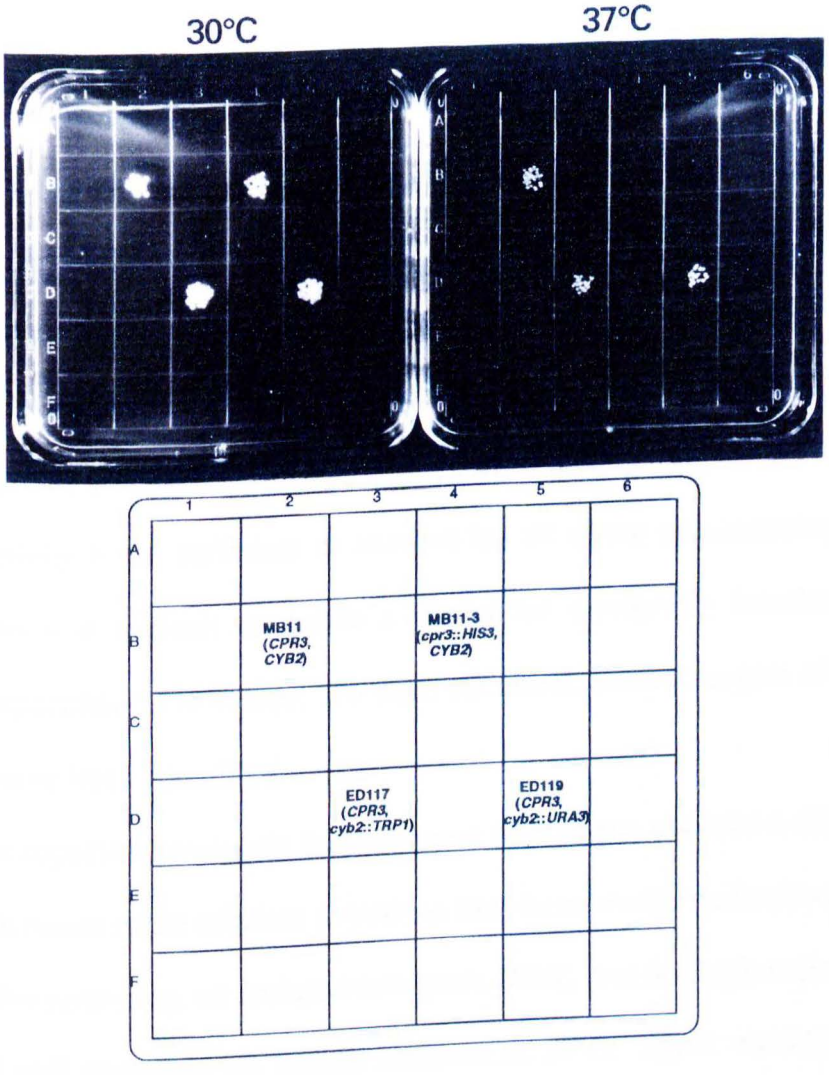

fumarase activity, units/mg X 10-2
100
80
60
40
20
strain:
MB11
ED117-1
ED119-1
flavocytochrome b2 activity, units/mg X 100
100
80
60
40
20
strain:
MB11
ED117-1
ED119-1

Figure 6C.


30°C
37°C
1
2
3
4
5
6
A
B
C
D
E
F
MB11
(CPR3,
CYB2)
MB11-3
(cpr3::HIS3,
CYB2)
ED117
(CPR3,
cyb2::TRP1)
ED119
(CPR3,
cyb2::URA3)

Chapter 4. Identification of physiological targets of Cpr3: Genetic Analysis of Cyclophilin Function: Identification of Loci That Suppress a Null Phenotype of the Yeast *CPR3* Gene.

Summary

The cyclophilins are a family of eukaryotic proteins that mediate the biological actions of the immunosuppressive drug cyclosporin A (CsA). The function of cyclophilins in cellular physiology is unknown. In a complex with CsA, cyclophilin inhibits signal transduction events required for T-lymphocyte activation. Cyclophilins also have a CsA-sensitive proline isomerase (PPIase) activity *in vitro*, suggesting that they may be involved in protein folding. However, inhibition of PPIase activity is not sufficient to account for all of the pharmacological effects of CsA, so it is difficult to derive a model for cyclophilin function based on existing experimental evidence. To date, no direct, natural targets of cyclophilin function have been identified.

We reported previously that the yeast *CPR3* gene encodes a mitochondrial cyclophilin required for efficient growth on non-fermentable carbon sources. After an extensive screening, we isolated one gene, *JEN1*, that is a high-copy suppressor of a *cpr3* null mutation on lactate medium at 37^0C. *JEN1* encodes a protein predicted to be similar to bacterial and eukaryotic carboxylic acid transporters. Deletion mutagenesis suggests that the *JEN1* gene product is involved in the transport of lactate. In a screen for single copy chromosomal suppressors, we

identified a dominant mutation in a second gene, *JEN2*, that suppresses the growth deficiency of *cpr3::HIS3* strains at both 30^0C and 37^0C. The specificity of suppression by *JEN2-1* suggests that *JEN2* might encode an *in vivo* target of Cpr3.

Introduction

The cyclophilins are a family of ubiquitous eukaryotic proteins defined by their ability to bind the immunosuppressive drug cyclosporin A (CsA) (Handschumacher, *et al.*, 1984). Despite intense research since the discovery of cyclophilins, their normal physiological functions remain enigmatic.

Two bodies of evidence suggest different functions for cyclophilins. First, as the receptors for CsA, cyclophilins are implicated in the inhibition of signal transduction in T-lymphocytes (reviewed in Sigal and Dumont, 1992). The current model for this inhibition is that CsA binds to cyclophilin to form a complex, that binds and inhibits calcineurin, a serine/threonine phosphatase necessary for the dephosphorylation of a subunit of nuclear factor of activated T-cells (NFAT). This dephosphorylation is required for the assembly of NFAT, which is in turn necessary for the antigen- or mitogen-stimulated transcriptional induction of lymphokine genes.

Second, cyclophilins possess peptidyl-prolyl *cis-trans* isomerase (PPIase) activity *in vitro*, which accelerates the rate of interconversion of *cis* and *trans* isomers of prolyl amide bonds (Fischer, *et al.*, 1989; Takahashi, *et al.*, 1989). Cyclophilin PPIase activity is inhibited by CsA. As proline isomerization can be a rate-limiting step of protein refolding *in vitro*, cyclophilins might be involved in *in vivo* protein folding. Support for this model comes from the finding that the cyclophilin homolog *ninaA* of *Drosophila melanogaster* is necessary for the efficient processing of an opsin in the secretory pathway of photoreceptor cells (Shieh, *et*

al., 1989; Stamnes, *et al.*, 1991; Colley, *et al.*, 1991). However, no direct interaction between Rh1 rhodopsin and the *ninaA* gene product has been demonstrated.

A synthetic analog of CsA inhibits PPIase activity without causing immunosuppression, suggesting that inhibition of PPIase is not sufficient to prevent T-cell activation (Sigal, *et al.*, 1991). Thus, the protein folding and signal transduction models of cyclophilins cannot be easily reconciled into a model for cyclophilin function. To date, no natural, direct targets of cyclophilin function have been identified *in vivo*.

We have been working to determine the physiological function of the cyclophilin encoded by the *CPR3* gene of *Saccharomyces cerevisiae*. Previously, we demonstrated that *CPR3* is essential for growth on lactate medium at elevated temperature (Davis, *et al.*, 1992). Subsequently, we determined that *CPR3* encodes a mitochondrial matrix CsA-binding protein needed for efficient mitochondrial function in general (E. S. D. and M. B. B., manuscript in preparation).

In adaptation to growth on non-fermentable carbon sources, the transcription of some nuclear cytochrome genes is induced by a complex signal transduction pathway involving communication between mitochondria and the nucleus (reviewed in Forsburg and Guarente, 1989). This induction is not significantly decreased in yeast lacking functional alleles of *CPR3* (E. S. D. and M. B. B., manuscript in preparation). In order to define more exactly the defect in mitochondrial function of *cpr3* mutants, we isolated genetic suppressors of their

growth phenotype.

In an exhaustive search for high-copy suppressors, we isolated two distinct groups of cloned fragments that alleviate growth deficiencies resulting from inactivation of *CPR3*. One of these is *CPR3*. The other, denoted *JEN1*, is capable of encoding a novel protein with homology to a family including bacterial and eukaryotic carboxylic acid transporters. We also isolated and characterized three strains harboring dominant nuclear mutations that suppress the phenotype of *cpr3::HIS3* strains at 37^0C. Two of these pseudo-revertants carried mutations that were specific for Cpr3, as they also suppressed the slow growth phenotype of the *cpr3::HIS3* mutation at 30^0C. *JEN2*, the gene defined by the mutation in one of these strains, is a strong candidate for encoding a natural physiological substrate or ligand for a cyclophilin, and may allow a molecular definition of cyclophilin function.

Materials and methods

Yeast strains and media. Strains MB11, MB11-3, MH272-3c and ED80-2 were described previously (Davis, *et al.*, 1992; Table 1A). Strains were normally cultured at 30^0C in either synthetic minimal media (0.67% Difco Bacto yeast nitrogen base, 2.0% glucose, and amino acid and nucleotide supplements as needed; Sherman, *et al.*, 1986) or YPD (2.0% peptone, 1.0% yeast extract, 2.0% glucose; Sherman, *et al.*, 1986). Strain growth was tested on YP supplemented with either 2.0% (w/v) glucose, 2.0% (w/v) D-, L-lactate, 0.1M L-lactate, or 3.0% glycerol. D-, L-lactate was initially prepared at 20%, the pH was adjusted to 4.6 with KOH, filter-sterilized, and added to autoclaved 1.1 X YP. L-lactate and glycerol media were prepared as described (Davis, et. al, 1992). For plates, 15 g L^{-1} agar was included. For spotting on plates, cells were grown in either YPD or synthetic minimal media to mid-logarithmic phase. Cell density was determined by plating on YPD plates at 30^0C prior to spotting.

Isolation of high-copy suppressors. YEp13 (Broach, *et al.*, 1979), and the yeast genomic library in YEp13 (5-20 kilobase-pair fragments generated by partial Sau3AI digest of DNA from wild-type strain AB320; Nasmyth and Reed 1980), were obtained from American Type Culture Collection as stocks in *Escherichia coli* strains C60 and K-12 RR1, respectively. Library DNA was isolated by plating *ca.* $1 \text{ X } 10^6$ ampicillin-resistant transformants. Cells were pooled, and plasmid DNA was extracted and purified by ethidium bromide-cesium chloride centrifugation as

described (Sambrook, *et al.*, 1989). Library DNA was introduced into strain ED80-2 by electroporation (Becker and Guarente, 1991). 3 X 10^5 Leu^+ yeast transformants were pooled and suspended in YPD. Approximately 1.2 X 10^5 cells, of which *ca.* 0.39 X 10^5 carry insert-containing plasmids (*ca.* 28 genome equivalents), were diluted in H_20, plated on YP supplemented with 2.0% D-, L-lactate, and incubated at 37^0C for seven days.

Deletion of *JEN1*. Strains ED279-1, ED279-2, ED280-1, ED281-1, ED282-1, ED282-2, ED283-1, ED287-1, and ED287-2 (Table 1E) were constructed by deletion of *JEN1* using the one-step gene disruption procedure (Rothstein, 1983): The 3.3 kb BamHI fragment of pESD38 was inserted into BamHI-digested pUC19, to create pESD45. pESD45 DNA was digested with BglII and SphI together, yielding fragments of 4.4, 1.3, and 0.2 kb. The 1.3 and 0.2 kb fragments include the desired region of *JEN1* to be deleted (see Figures 3 and 5A). The 4.4 kb fragment, which includes all of the pUC19 DNA flanked by 0.4kb and 1.3kb of *JEN1* DNA, was gel-purified by standard methods (Sambrook, *et al.*, 1989). A 1.1 kb XbaI fragment containing the *S. cerevisiae URA3* gene (Rose, *et al.*, 1984; positions -227 to +943 of the protein coding region) was inserted into the XbaI site of plasmid pUC19, excised with BamHI and SphI, and gel purified. This *URA3*-containing fragment was then ligated to the 4.4 kb BglII-SphI fragment from pESD45, to yield pESD48. A *jen1::URA3* construct suitable for recombination at the *JEN1* locus was liberated from circular pESD48 by digestion with restriction endonuclease Bam HI (see Figure 5A). This digest yielded a

pUC19 DNA fragment, and a *URA3*-containing fragment flanked by 0.4kb and 1.3kb of *JEN1* DNA. BamHI-digested pESD48 was introduced into yeast by electroporation as described (Becker and Guarente, 1991).

To confirm deletion at the *JEN1* locus, polymerase chain reaction amplification (Saiki, *et al.*, 1985) was performed as follows: Yeast genomic DNA (*ca.* 10 ng) was mixed in 100 μl reaction volumes containing 200 μM each dCTP, dGTP, dATP, and dTTP; 1 μM oligonucleotide primers JEN101 (5′ GCA TTA GAA CGT TAC CTG GTC 3′; positions -269 to -249) and ED43C1 (5′ CAA TCA TCT TCA CTG AAG CAC 3′; positions +1,804 to +1,784); and 5.0 units *Thermus aquaticus* DNA polymerase (Boehringer Mannheim). 30 cycles were performed, using temperature steps of 94^{0}C, 1.5 min.; 50^{0}C, 3.0 min.; and 72^{0}C, 2.5 min. PCR products were analyzed by agarose gel electrophoresis in the presence of ethidium bromide as described (Sambrook, *et al.*, 1989). Using these primers in a PCR reaction, the *JEN1*$^+$ allele yielded a 2.1kb product, while the *jen1::URA3* allele yielded a 1.7kb product.

Northern analysis of *JEN1* expression. Strains MH272-3c, ED282-1, and ED282-2 were cultured in YP containing 10% (w/v) glucose at 30^{0}C to an A_{600} = 0.5-0.8. One aliquot was used for RNA preparation. Another aliquot was centrifuged, resuspended in 0.5ml H_2O, and used to inoculate 50ml YP supplemented with 2.0% D-, L- lactate or 3.0% glycerol in baffled flasks at an A_{600} = 0.03. Cultures were grown for 21 hours at 30^{0}C, followed by RNA extraction. Duplicate Northern blots were prepared as described below. Each was hybridized with a

different, gel-purified BamHI-SphI *JEN1* fragment labeled with ^{32}P, one of 2.0 kilobases (positions -672 to +1,316 of the predicted protein coding region), and one of 1.3 kilobases (positions +1,317 to +2,563).

Miscellaneous. All cloning manipulations, yeast genomic and plasmid DNA extractions, RNA extractions, blotting, hybridization, washing, and autoradiography were performed by standard methods (Sherman, *et al.*, 1986; Sambrook, *et al.*, 1989). Sporulation and tetrad dissection were performed as described (Sherman, *et al.*, 1986; Sherman and Hicks, 1991). DNA probes were labeled by random priming (Sambrook, *et al.*, 1989), and purified over Sephadex G-50 columns. PCR of *CPR3* alleles was performed using oligonucleotides Y-2A and Y-2B as described (Davis, *et al.*, 1992). DNA sequencing was performed on ethidium bromide-cesium chloride purified, double stranded plasmid DNA by the dideoxy chain-termination method (Sanger, *et al.*, 1977) using a kit (United States Biochemicals).

Results

We previously demonstrated that yeast strains carrying null mutations in the *CPR3* gene are impaired for growth on non-fermentable carbon sources. These mutants are incapable of growth on lactate medium at 37^{0}C, allowing selection of revertants (Davis, *et al.*, 1992). In order to define the function of Cpr3 in mitochondria more precisely, we applied this selection to isolate both high-copy and single-copy suppressors of the *cpr3::HIS3* phenotype.

Isolation of *JEN1*, a high-copy suppressor gene. We carried out an exhaustive search of a wild-type genomic library constructed in the high-copy vector, YEp13, for fragments capable of suppressing the *cpr3* null phenotype on lactate medium at 37^{0}C. Plating approximately 28 genome equivalents of this library (see Materials and Methods) in strain ED80-2 on lactate medium at 37^{0}C yielded 15 revertant colonies.

Plasmid DNA from 13 of these candidates had overlapping restriction patterns consistent with the known restriction map of *CPR3* (Davis, *et al.*, 1992). This identity was confirmed by Southern hybridization (not shown). The other two plasmids, pESD33 and pESD36, from revertant colonies (ED252 and ED253, repectively; see Table 1B) that grew much more poorly, had inserts of *ca.* 8.5 kb; these inserts had similar restriction maps, and did not hybridize with *CPR3*. pESD33, as well as one of the *CPR3*-containing plasmids, pESD34, was chosen for further analysis.

To ensure that the reversion was encoded by the plasmid and not by a chromosomal mutation, we did two experiments. First, we re-introduced plasmids pESD33, pESD34, and YEp13 into strain ED80-2. Second, we cured the original revertant isolates of their plasmids by screening for leucine auxotrophy. Leu^+ transformants and leucine-auxotrophic segregants were tested for their ability to grow on lactate medium at 37^0C. The result of this test is shown in Figure 1.

All strains carrying pESD33 and pESD34 (*CPR3*) were able to grow on lactate medium at 37^0C, but leucine auxotrophs derived from the original revertant isolates could not. This indicates that the plasmids pESD33 and pESD34 (*CPR3*), and not a chromosomal mutation, permits the *cpr3::HIS3* mutant strain to grow on lactate medium at 37^0C. As expected, strains carrying YEp13 (no insert) behaved like ED80-2 (*cpr3::HIS3*).

To localize the suppressing gene on the pESD33 insert, we constructed five subclones, depicted schematically in Figure 2. Digestion of pESD33 with SphI yielded two fragments, one of 4.4kb and another of 2.6kb, whereas digestion with Bam HI released a 3.3kb fragment. All of these fragments were isolated and cloned into YEp13. In addition, Bam HI- or SphI digests of pESD33 were re-circularized without internal fragments and isolated as circular plasmids.

Two independent isolates of each of the 5 subclones were transformed into strain ED80-2, and Leu^+ transformants were tested for their ability to grow on lactate medium at 37^0C. Only the 3.3 kb BamHI fragment in YEp13 suppressed the phenotype of the *cpr3::HIS3* mutation (Figure 2). The phenotype resulting from this plasmid, pESD38, was similar to that of pESD33 (not shown). This

result demonstrated that the high-copy suppressor function lies within this Bam HI fragment and spans an SphI site.

Cleavage of the 3.3 kb BamHI fragment with SphI generated two fragments, of 1.3 kb and 2.0 kb, which were cloned into pUC19 and sequenced. The sequence contains one large open reading frame (ORF), capable of encoding a protein of 616 amino acids (Figure 3). In FASTA searches (Genetics Computer Group; Devereaux, *et al.*, 1984), neither DNA from the long ORF nor flanking DNA had significant similarity with any sequences in the Genbank and EMBL data bases.

A FASTA search of the SwissProt database revealed that the protein predicted for the long ORF has significant similarity to a family of proteins initially defined as a group of bacterial carboxylic acid transporters (Figure 4; Culham, *et al.*, 1993). This family also includes a protein encoded by a gene for methotrexate resistance in the yeast, *Candida albicans* (Fling, *et al.*, 1991), and a lactate/pyruvate transporter from Chinese hamster ovary cells (Garcia, *et al.*, 1994). Of highest similarity to the predicted protein is the product of the *Escherichia coli proP* gene, a transporter essential for proline and betaine utilization (20.6% amino acid identity). We have designated this gene *JEN1*.

We next characterized the nature of the suppression by *JEN1*, to determine whether the predicted protein interacts directly with Cpr3. This is complicated by the need for two environmental stresses to make the requirement for Cpr3 absolute: One stress is a limiting, non-fermentable carbon source: Increasing the concentration of lactate in the medium can weakly suppress the growth phenotype

of the *cpr3::HIS3* mutation (E. S. D. and M. B. B., unpublished observations). The other stress is elevated (37^0C) temperature: Mutations that suppress temperature sensitivity in *S. cerevisiae* are known (McCusker, *et al.*, 1994). While it is possible that these stresses are related to the function of Cpr3, *e. g.* Cpr3 may effect resistance to high temperature, we cannot be certain of this. If we consider these stresses to be independent, then suppression may act indirectly, by conferring temperature resistance for example, rather than by directly affecting the *cpr3* defect.

Two factors may help distinguish between direct and indirect suppression. First, we expect the products of direct suppressors to be mitochondrial proteins. Second, a direct suppressor may also stimulate the growth of *cpr3::HIS3* mutants on non-fermentable carbon sources at 30^0C. We considered the relationship of the *JEN1* gene product to Cpr3 by these criteria.

The first 21 amino acids of the predicted *JEN1* protein is rich in basic and hydroxylated residues, a feature of proteins targeted to mitochondria (reviewed in Roise and Schatz, 1988). However, this presumptive leader sequence also possesses three acidic residues, which are generally not found in mitochondrial targeting signals. In addition, a helical wheel projection (not shown) of the presumptive leader sequence shows that an α-helix formed by this region would not be strongly amphipathic. Therefore, predictions of the subcellular localization of the apparent *JEN1* gene product based on sequence analysis are inconclusive.

In order to determine if the suppression mediated by *JEN1* overexpression is direct, we next measured the generation times of MH272-3c, ED80-2, and

ED255-2 in lactate medium at 30^{0}C. ED255-2 carries pESD38-2, a plasmid containing the *JEN1* 3.3kb Bam HI fragment (Figure 2). Table 2 shows that plasmid pESD38-2 did not consistently decrease the generation time of the *cpr3* mutant on lactate at 30^{0}C.

To verify that the sequences of the *JEN1* ORF are indeed transcribed, we isolated RNA from yeast grown in rich medium supplemented with either glucose, glycerol, or lactate (Figure 5B). On Northern blots, each of the two labeled BamHI/SphI fragments spanning *JEN1* hybridize with an mRNA of *ca.* 2.0 kilobases, consistent with sequence prediction. Most significantly, this transcript was abundant in RNA from yeast grown on lactate, but very rare or absent from cells grown on glucose or glycerol. This result suggested that the *JEN1* gene product is involved in lactate metabolism, possibly as a lactate transporter.

To assess the function of the *JEN1* gene product, a deletion allele (*jen1::URA3*) was constructed. We removed a 1,553 base BglII-SphI DNA fragment that includes the amino terminal 439 residues of the predicted protein sequence, and replaced it with the *S. cerevisiae URA3* gene (Figure 6A). Transformants were obtained both in diploid strains ED284-1 (*CPR3/CPR3*) and CMY214 (*CPR3/CPR3*), and in haploid strains MH272-3c (*CPR3*), ED80-2 (*cpr3::HIS3*), MB11 (*CPR3*), and MB11-3 (*cpr3::HIS3*). Deletion at the *JEN1* locus was confirmed by polymerase chain reaction (PCR) analysis (not shown). Northern blotting indicated that this deletion abolishes *JEN1* mRNA expression in haploid strains (Figure 5B). Therefore, *JEN1*, either alone or in combination with *CPR3*, is not essential for viability under standard growth conditions (glucose

at 30^{0}C).

We tested the growth of haploid strains carrying the *jen1::URA3* allele on rich medium supplemented with either glucose, glycerol, or L-lactate. Strains plated on these media were incubated at 30^{0}C and at 37^{0}C. No growth phenotype was observed for these strains on glucose or glycerol media at either temperature (not shown). However, two independent, *CPR3*$^+$ *jen1::URA3* derivatives of MH272-3c, ED282-1 and ED282-2, formed smaller colonies than wild-type on rich medium supplemented with L-lactate at 37^{0}C, indicating slower growth (Figure 5C). A similar phenotype was observed for growth of the *CPR3*$^+$ *jen1::URA3* strain ED280-1, a derivative of MB11, on synthetic lactate medium at 30^{0}C (not shown). This phenotype is reminiscent of that of *cyb2* (mitochondrial lactate dehydrogenase) mutants, which exhibit normal growth on media supplemented with the isomeric mixture D-, L-lactate, but are impaired for growth on YP media supplemented with L-lactate at 37^{0}C (unpublished observations). Presumably, both *cyb2* and *jen1* mutants are impaired in the metabolism of L-lactate, but are still able to metabolize D-lactate, or other carbon sources in the medium (such as amino acids). These results are consistent with the possibility that *JEN1* encodes a lactate transporter.

Single-copy suppressors. Despite the extensive search for high copy suppressors, we identified only one, *JEN1*. To complement this high copy screen, we sought to identify chromosomal (single copy) suppressors of the *cpr3::HIS3* phenotype. We identified three spontaneously-arising, revertant colonies of the *cpr3::HIS3* strain

MB11-3 on lactate medium at 37°C (Figure 6A). Polymerase chain reaction revealed that ED259-1, ED260-1, and ED261-1 still contained the *cpr3::HIS3* allele of strain MB11-3, and no other *CPR3* alleles (not shown). These pseudo-revertants carried plasmid constructs from diploid, pseudo-revertant strain genomic libraries in the yeast centromeric plasmid pRS314 (Sikorsky and Hieter, 1989). Restriction enzyme analysis showed that the plasmid from ED259-1 (pESD49) did not contain an insert, whereas the plasmids from ED260-1 (pESD50) and ED261-1 (pESD51) did (not shown). Re-introduction of these plasmids to MB11-3 did not stimulate growth on lactate medium at 37°C (not shown). These results indicated that the suppressors in these strains are not plasmid-encoded.

We next tested the suppressor alleles for dominance. We mated strains ED259-1, ED260-1, and ED261-1 to ED134-1, a *cpr3::HIS3* strain of opposite mating type. ED134-1 does not grow on lactate medium at 37°C (not shown). Figure 6A shows the results of testing the growth of these diploids on lactate medium at 30°C and 37°C. As expected, control diploids ED179 (*CPR3*$^+$/*CPR3*$^+$) and ED180 (*CPR3*$^+$/*cpr3::HIS3*) were able to grow on lactate at 37°C, while ED181 (*cpr3::HIS3/cpr3::HIS3*) did not. The diploids generated by mating ED259-1, ED260-1, and ED261-1 (ED262, ED263, and ED264, respectively) all grew as well, indicating that the suppressor genes in the original pseudo-revertant strains are dominant.

Diploid strains ED181, ED262, ED263, and ED264 were sporulated, and haploid progeny dissected from two asci of each meiosis were tested for their

ability to grow on lactate medium at 37^{0}C. At least one of the two asci from each pseudo-revertant diploid displayed 2:2 segregation of the suppressors, consistent with each of the pseudo-revertants carrying dominant, nuclear mutations (Table 3). As expected, none of the spore progeny from ED181 grew on lactate medium at 37^{0}C (Table 3).

As discussed above, we characterized the relationship of these suppressors to *CPR3* by measuring the growth rate of pseudo-revertant strains in liquid D-, L-lactate medium at 30^{0}C. Table 4 shows the results of this growth assay. Of the three pseudo-revertant strains, only strains ED260-1 and ED261-1 consistently grew faster than the parent strain, MB11-3. The generation time, 11% faster than that of MB11-3, is 12% slower than that of MB11. These results suggest that ED260-1 and ED261-1 contain *bona fide* second-site suppressor mutations of the *cpr3::HIS3* deficiency.

ED261-1 was cured of its centromeric plasmid (pESD51) by screening for tryptophan auxotrophs, after growth under non-selective conditions (YPD medium), to yield ED261-2 and ED261-3. The phenotype of these derivatives on lactate plates at 37^{0}C is indistinguishable from ED261-1 (not shown). Deletion of *JEN1* in ED261-2 did not affect the phenotype, demonstrating that this suppressor is not allelic to *JEN1* (Figure 6B). We designated this suppressor *JEN2-1*, a dominant allele of *JEN2*$^+$.

Thus, we have identified three *cpr3::HIS3* strains carrying dominant nuclear mutations that allow growth on lactate medium at 37^{0}C. The *JEN2-1* mutation, in ED261-1, also suppresses the *cpr3::HIS3* phenotype on lactate at 30^{0}C, and

therefore displays traits expected of a genuine suppressor of the *cpr3::HIS3* deficiency. We do not yet know if the suppressor mutation in ED260-1 is allelic with either *JEN1* or *JEN2*.

Discussion

We are interested in determining the function of the cyclophilins, the intracellular receptors for cyclosporin A. Although, as described above, evidence suggestive of roles in signal transduction and in protein folding has accumulated, the physiological roles of the cyclophilins have remained elusive.

Previously, we demonstrated that the yeast *CPR3* gene encodes a cyclophilin required for efficient growth at elevated temperature on non-fermentable carbon sources, particularly lactic acid (Davis, *et al.*, 1992). We have since determined that *cpr3* mutants are impaired for growth at 30^0C as well (E. S. D. and M. B. B., manuscript in preparation). Growth on non-fermentable carbon sources requires the transcriptional induction of nuclear genes encoding cytochromes and other mitochondrial proteins (Forsburg and Guarente, 1989). Transcriptional induction of genes encoding iso-1-cytochrome c and flavocytochrome b_2, a mitochondrial lactate dehydrogenase, is not significantly decreased in strains carrying the null allele *cpr3::HIS3*. Therefore, the growth defect of these strains is not in the signal transduction events governing cytochrome gene expression (E. S. D. and M. B. B., manuscript in preparation).

Three experiments show that the defect in mitochondrial function in *cpr3* mutants is not specific for lactate metabolism (E. S. D. and M. B. B., manuscript in preparation). First, at 30^0C, *cpr3::HIS3* mutants have approximately normal levels of flavocytochrome b_2, which was reported to be essential for lactate metabolism in *S. cerevisiae*. Second, *cyb2* null mutants show no growth phenotype

on rich medium supplemented with D-, L-lactate at 37^0, conditions that do not permit growth of *cpr3* mutants. Third, the growth defect of *cpr3* mutants was also observed on rich medium unsupplemented with carbon source. Together, these results demonstrate that Cpr3 plays a general role in mitochondrial function.

To date, direct targets of cyclophilin function have not been identified. To define more precisely the role of Cpr3 in yeast mitochondrial function, we undertook a genetic approach: Identifying second-site genetic loci that suppress, by either mutation or overexpression, the mitochondrial deficiency of *cpr3::HIS3* strains.

In a search for suppressors from *ca.* 28 genome equivalents of a wild-type library cloned into a high-copy plasmid vector, we identified 15 revertant colonies. Of these, 13 carried plasmids with *CPR3*-containing inserts (complementation of the growth defect of *cpr3::HIS3* mutants by *CPR3* was expected from this library). The two other plasmids, which weakly rescue the growth deficiency of *cpr3::HIS3* strains on lactate medium at 37^0C, carried a novel gene, *JEN1*. The predicted protein of the *JEN1* gene is similar to a protein family that includes bacterial carboxylic acid transporters, a methotrexate resistance protein from the yeast, *C. albicans*, and a mammalian lactate/pyruvate transporter.

JEN1 mRNA is very abundant in cells grown on lactate medium, but not in cells grown on glucose or glycerol. Also, haploid strains carrying the deletion allele *jen1::URA3* are impaired for growth on synthetic medium supplemented with L-lactate at 30^0C, and on rich medium supplemented with L-lactate at 37^0C. No growth phenotype for *jen1::URA3* strains was observed for growth on glycerol

at either temperature. Taken together, these results suggest that the *JEN1* gene product plays a role in lactate metabolism, probably as a transporter.

The similarity of the predicted *JEN1* gene product to bacterial and yeast carboxylic acid transporters is intriguing, as proline isomerases previously have been implicated in the function of transporters and ion channels. CsA inhibits the Ca^{2+}-induced swelling of rat liver and heart mitochondria. This effect is thought to be mediated by inhibition of an adenine nucleotide transporter (Halestrap and Davidson, 1990). CsA inhibits the surface expression of ligand-gated ion channels injected into *Xenopus leavis* oocytes (Helakar, *et al.*, 1994); this effect is suppressed by high levels of expression of an injected rat cyclophilin gene.

Finally, yeast auxotrophic for tryptophan and histidine are sensitive to FK-506, a macrolide antibiotic that inhibits the activity of a distinct PPIase family, the FK-506 binding proteins (FKBPs; Heitman, *et al.*, 1993). This phenotype is suppressed by overexpression of two proteins with similarity to amino acid transporters, and FK-506 inhibits amino acid uptake by yeast cells (Heitman, *et al.*, 1993). These results suggest that an FKBP is required for the activity of amino acid transporters. However, the FKBP involved in this amino acid transport has not been identified.

The biochemical relationship between the *CPR3* and *JEN1* gene products is uncertain. As Cpr3 is a mitochondrial protein (E. S. D. and M. B. B., manuscript in preparation), we expect proteins that directly interact with Cpr3 to be mitochondrial as well. Analysis of the predicted *JEN1* protein sequence reveals that a leader sequence of 21 amino acids possesses some, but not all, of the

features of sequences known to direct localization to mitochondria. Thus, whether the predicted *JEN1* protein is localized to the cell or mitochondrial membranes is an open question. Similarly, the observation that *JEN1* overexpression does not suppress the growth phenotype of *cpr3::HIS3* strains at 30^0C is inconclusive as to whether Jen1 interacts directly with Cpr3. Possibly, Jen1 is only one of several targets of Cpr3 function.

The dearth of high-copy suppressors was striking, indicating that overexpression of no other single gene is sufficient to allow growth of *cpr3::HIS3* mutants on lactate medium at 37^0C. A number of scenarios consistent with this result are imaginable. If Cpr3 aids the folding of a large number of mitochondrial proteins, overexpression of any one of them (other than possibly Jen1) might not be sufficient to permit growth of *cpr3* mutants under restrictive conditions. Alternatively, if Cpr3 aids the folding of one particular protein, its overexpression might not increase the total amount of properly folded protein in mitochondria in *cpr3* mutants.

To complement our search for high-copy suppressors of the *cpr3::HIS3* phenotype, we selected spontaneous pseudo-revertants on lactate medium at 37^0C. These mutations arose at a frequency of *ca.* 2×10^{-5}. Three such pseudo-revertants were isolated, carrying dominant nuclear suppressor mutations. Two of the pseudo-revertants, ED260-1 and ED261-1, carried mutations that consistently suppressed the slow growth phenotype of the *cpr3::HIS3* allele on lactate medium at 30^0C, while the other one, ED259-1 did not. It is possible that the mutation in ED259-1 is suppressing the inherent temperature sensitivity of

laboratory yeast (McCusker, *et al.*, 1994), rather than a Cpr3 deficiency directly. The suppressor in ED261-1 is not allelic with *JEN1*, so we have designated its wild-type allele *JEN2*$^{+}$.

Our results suggest that *JEN2* is a strong candidate for encoding a genuine biochemical target of Cpr3. The next steps in our analysis of cyclophilin function will be the cloning and characterization of *JEN2*.

Most genes in yeast appear not to be essential for viability. In order to analyze them, it may be necessary to use environmental stresses to uncover a strong phenotype. The physiology of yeast growing under stress will complicate such analyses, as it has our analysis of *cpr3::HIS3* suppressors, but this approach should still yield useful results.

References

Becker, D. M. & Guarente, L. (1991). High-efficiency transformation of yeast by electroporation. *Meth. Enzymol.* **194**, 182-187.

Broach, J. R., Strathern, J. N., & Hicks, J. B. (1979). Transformation in yeast: Development of a hybrid cloning vector and isolation of the *CAN1* gene. *Gene* **8**, 121-133.

Colley, N. J., Baker, E. K., Stamnes, M. A., & Zuker, C. S. (1991). The cyclophilin homolog *ninaA* is required in the secretory pathway. *Cell* **67**, 255-263.

Culham, D. E., Lasby, B., Marangoni, A. G., Milner, J. L., Steer, B. A., van Nues, R. W., & Wood, J. M. (1993). Isolation and sequencing of *Escherichia coli* gene *proP* reveals unusual structural features of the osmoregulatory proline/betaine transporter, ProP. *J. Mol. Biol.* **229**, 268-276.

Davis, E. S., Becker, A., Heitman, J., Hall, M. N., & Brennan, M. B. (1992). A yeast cyclophilin gene essential for lactate metabolism at high temperature. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 11169-11173.

Devereux, J., Haeberli, P., & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**, 387-395.

Fischer, G., Wittman-Liebold, B., Lang, K., Kiefhaber, T., & Schmid, F. X. (1989). cyclophilin and peptidyl-prolyl *cis-trans* isomerase are probably identical proteins. *Nature* **337**, 476-478.

Fling, M. E., Kopf, J., Tamarkin, A., Gorman, J. A., Smith, H. A., & Koltin, Y. (1991). Analysis of a *Candida albicans* gene that encodes a novel mechanism for resistance to benomyl and methotrexate. *Mol. Gen. Genet.* **227**, 318-329.

Forsburg, S. L. & Guarente, L. (1989). Communication between mitochondria and the nucleus in regulation of cytochrome genes in the yeast *Saccharomyces cerevisiae*. *Ann. Rev. Cell Biol.* **5**, 153-180.

Garcia, C. K., Goldstein, J. L., Pathak, R. K., Anderson, R. G. W., & Brown, M. S. (1994). Molecular characterization of a membrane transporter for lactate, pyruvate, and other monocarboxylates: Implications for the Cori cycle. *Cell* **76**, 865-873.

Halestrap, A. P. & Davidson, A. M. (1990). Inhibition of Ca^{2+}-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl *cis-trans* isomerase and preventing it interacting with the adenine nucleotide translocase. *Biochem. J.* **268**, 153-160.

Handschumacher, R. E., Harding, M. W., Rice, J., Drugge, R. J., & Speicher, D. W. (1984). Cyclophilin: A specific cytosolic binding protein for cyclosporin A. *Science* **226**, 544-547.

Heitman, J., Koller, A., Kunz, J., Henriquez, R., Schmidt, A., Movva, N. R., & Hall, M. N. (1993). The immunosuppressant FK506 inhibits amino acid import in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13**, 5010-5019.

Helakar, S. A., Char, D., Neff, S., & Patrick, J. (1994). Prolyl isomerase requirement for the expression of functional homo-oligomeric ligand-gated ion channels. *Neuron* **12**, 179-189.

Kim, C. M., Goldstein, J. L., & Brown, M. S. (1992). cDNA cloning of MEV, a mutant protein that facilitates cellular uptake of mevalonate, and identification of the point mutation responsible for its gain of function. *J. Biol. Chem.* **267**, 23113-23121.

McCusker, J. H., Clemons, K. V., Stevens, D. A., & Davis, R. W. (1994). Genetic characterization of pathogenic *Saccharomyces cerevisiae* isolates. *Genetics* **136**, 1261-1269.

Nasmyth, K. A. & Reed, S. I. (1980). Isolation of genes by complementation in yeast: Molecular cloning of a cell-cycle gene. *Proc. Natl. Acad. Sci. U. S. A.* **77**,

2119-2123.

Roise, D. & Schatz, G. (1988). Mitochondrial presequences. *J. Biol. Chem.* **263**, 4509-4511.

Rose, M., Grisafi, P., & Botstein, D. (1984). Structure and function of the yeast *URA3* gene: Expression in *Escherichia coli*. *Gene* **29**, 113-124.

Rothstein, R. J. (1983). One-step gene disruption in yeast. *Methods Enzymol.* **152**, 481-504.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sherman, F. & Hicks, J. (1991). Micromanipulation and dissection of asci. *Methods Enzymol.* **194**, 21-37.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., & Arnheim, N. (1985). Enzymatic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of sickle-cell anemia. *Science* **230**, 1350-1354.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463-5467.

Seol, W. & Shatkin, A. J. (1991). The *Escherichia coli kgtP* gene encodes an α-ketoglutarate transporter. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 3802-3806.

Sherman, F., Fink, G. R., & Hicks, J. B. (1986). Laboratory course manual for methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Shieh, B-H., Stamnes, M. A., Seavello, S., Harris, G. L., & Zuker, C. S. (1989). The *ninaA* gene required for visual transduction in *Drosophila* encodes a homolog of cyclosporin A-binding protein. *Nature* **338**, 67-70.

Sigal, N. H., Dumont, F., Durette, P., Siekierka, J. J., Peterson, L., Rich, D. H., Dunlap, B. E., Staruch, M. J., Melino, M. R., Koprak, S. L., Williams, D., Witzel, B., & Pisano, J. M. (1991). Is cyclophilin involved in the immunosuppressive and nephrotoxic action of cyclosporin A? *J. Exp. Med.* **173**, 619-628.

Sigal, N. H. & Dumont, F. J. (1992). Cyclosporin A, FK-506, and rapamycin: Pharmacologic probes of lymphocyte signal transduction. *Ann. Rev. Immunol.* **10**, 519-560.

Sikorski, R. S. & Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19-27.

Stamnes, M. A., Shieh, B-H., Chuman, L., Harris, G. L., & Zuker, C. S. (1991). The cyclophilin homolog *ninaA* is a tissue-specific integral membrane protein required for the proper synthesis of a subset of *Drosophila* rhodopsins. *Cell* **65**, 219-227.

Susatsu, M., Misra, T. K., Chu, L., Laddaga, R., & Silver, S. (1985). Cloning and DNA sequence of a plasmid-determined citrate utilization system in *Escherichia coli*. *J. Bacteriol.* **164**, 983-993.

Takahashi, N., Hayano, T., & Suzuki, M. (1989). Peptidyl-prolyl *cis-trans* isomerase is the cyclosporin A-binding protein cyclophilin. *Nature* **337**, 473-475.

van der Rest, M. E., Schwarz, E., Oesterhelt, O., & Konings, W. N. (1990). DNA sequence of a citrate carrier of *Klebsiella pneumoniae*. *Eur. J. Biochem.* **189**, 401-407.

Table 1. Yeast Strains.

A. Basic strains

Strain	Genotype
MH272-3c	*MAT***a** *HML***a** *trp1-1 his3 leu2-3,112 ura3-52 rme1*
ED80-2	MH272-3c *cpr3::HIS3*
MH272-1d	*MATα HML***a** *trp1-1 his3 leu2-3,112 ura3-52 rme1*
ED284	*MAT***a***/MATα* (MH272-3c X MH272-1d)
CMY214	*MAT***a***/MATα ade2-101/ade2-101 trp1-Δ1 ura3-52/ura3-52 his3-Δ200/his3-Δ200 lys2-801/lys2-801 CAN1/can1*
MB11	*MATα ade2-101 trp1-Δ1 ura3-52 his3-Δ200 lys2-801 can1*
ED127-1	MB11 [pRS314 *TRP1 CEN6 ARSH4*]
MB11-3	MB11 *cpr3::HIS3*
ED128-1	MB11-3 [pRS314 *TRP1 CEN6 ARSH4*]
ED133-1	*MAT***a** *ade2-101 trp1-Δ1 ura3-52 his3-Δ200 lys2-801* [pRS316 *URA3 CEN6 ARSH4*]
ED134-1	ED133-1 *cpr3::HIS3*

B. Strains used for isolation and subcloning of *JEN1*

Strain	Genotype
ED249*	ED80-2 [YEp13 *LEU2*]

Table 1B (continued)

Strain	Genotype
ED249-1	ED80-2 [YEp13 *LEU2*]
ED249-2	ED80-2 [YEp13 *LEU2*]
ED250*	ED80-2 [pESD34 *LEU2 CPR3*]
ED250-1	ED80-2 [pESD34 *LEU2 CPR3*]
ED250-2	ED80-2 [pESD34 *LEU2 CPR3*]
ED250-3	ED250* *leu2*
ED250-4	ED250* *leu2*
ED252*	ED80-2 [pESD33 *LEU2 JEN1*]
ED252-1	ED80-2 [pESD33 *LEU2 JEN1*]
ED252-2	ED80-2 [pESD33 *LEU2 JEN1*]
ED252-3	ED252* *leu2*
ED252-4	ED252* *leu2*
ED253*	ED80-2 [pESD36@ *LEU2 JEN1*]
ED254-1	ED80-2 [pESD37-1§ *LEU2*]
ED254-2	ED80-2 [pESD37-2§ *LEU2*]
ED255-1	ED80-2 [pESD38-1§ *LEU2 JEN1*]
ED255-2	ED80-2 [pESD38-2§ *LEU2 JEN1*]
ED256-1	ED80-2 [pESD39-1§ *LEU2*]
ED256-2	ED80-2 [pESD39-2§ *LEU2*]
ED257-1	ED80-2 [pESD40-1§ *LEU2*]

Table 1B (continued)

Strain	Genotype
ED257-2	ED80-2 [pESD40-2[§] *LEU2*]
ED258-1	ED80-2 [pESD41-1[§] *LEU2*]
ED258-2	ED80-2 [pESD41-2[§] *LEU2*]

[*]Original isolate of strain.

[@]This plasmid, which has a similar restriction map to pESD33, is described in Results.

[§]These plasmids are described in the legend to Figure 2.

C. Pseudo-revertants derived from MB11-3

Strain	Genotype
ED259-1	MB11-3 [pESD49[¶] *TRP1 CEN6 ARSH4*]
ED260-1	MB11-3 [pESD50[¶] *TRP1 CEN6 ARSH4*]
ED261-1	MB11-3 *JEN2-1* [pESD51[¶] *TRP1 CEN6 ARSH4*]
ED261-2	ED261-1 *trp1*
ED261-3	ED261-1 *trp1*

[¶]Plasmids pESD49, pESD50, and pESD51, which are derivatives of pRS314 (Sikorsky and Hieter, 1989), are described in Results.

Table 1 (continued)

D. Diploids used to establish dominance of single-copy suppressors

Strain	Genotype
ED179-1	*MAT***a**/*MATα* (ED127-1 X ED133-1)
ED180-1	*MAT***a**/*MATα CPR3*$^{+}$/*cpr3::HIS3* (ED127-1 X ED134-1)
ED181-1	*MAT***a**/*MATα cpr3::HIS3/cpr3::HIS3* (ED128-1 X ED134-1)
ED262-1	*MAT***a**/*MATα cpr3::HIS3/cpr3::HIS3* (ED259-1 X ED134-1)
ED263-1	*MAT***a**/*MATα cpr3::HIS3/cpr3::HIS3* (ED259-1 X ED134-1)
ED264-1	*MAT***a**/*MATα cpr3::HIS3/cpr3::HIS3 JEN2-1/jen2*$^{+}$ (ED259-1 X ED134-1)

E. Strains carrying deletion alleles of *JEN1*

Strain	Genotype
ED279-1	CMY214 *JEN1/jen1::URA3*
ED280-1	MB11 *jen1::URA3*
ED281-1	MB11-3 *jen1::URA3*
ED281-2	MB11-3 *jen1::URA3*
ED282-1	MH272-3c *jen1::URA3*

Table 1E (continued)

Strain	Genotype
ED282-2	MH272-3c *jen1::URA3*
ED283-1	ED80-2 *jen1::URA3*
ED285	ED284 *JEN1*$^{+}$*/jen1::URA3*
ED287-1	ED261-2 *jen1::URA3*
ED287-2	ED261-2 *jen1::URA3*

Table 2. Yeast generation times: Overexpression of *JEN1*.

Strain	Generation time at 28^{0}C (hours)
MH272-3c (*CPR3*$^+$)	3.1
ED80-2 (*cpr3*)	4.5
ED255-2 (*cpr3*, pESD38 [*LEU2*, *JEN1*])	4.4

pESD38 is a derivative of YEp13 containing a 3.3 kb Bam HI fragment that carries *JEN1* (see Table 1 and Figure 2). All strains were cultured in synthetic minimal media containing 2.0% glucose overnight at 28^{0}C. Cells were diluted into 50 ml liquid YP supplemented with 2.0% D-, L-lactate in 0.5L baffled flasks, to an A_{600} = 0.03. Cultures were incubated with 300 rpm shaking at 28^{0}C. The first optical density measurement took place after eight hours, and then once every four hours. Generation times were calculated from optical density measurements of cultures from 20-24 hours after inoculation. Experiment was performed three times. Representative data from one trial are shown.

Table 3. Segregation of suppressors.

Strain	tetrad	# of lac+ spores at 37°C	# of lac- spores at 37°C
ED181-1	1	0	4
	2	0	4
ED262	1	2	2
	2	0	4
ED263	1	1	3
	2	2	2
ED264	1	2	2
	2	2	2

All strains listed in the table lack functional alleles of *CPR3*. Cultures were grown overnight in YP containing 2.0% glucose at 30°C. Growth of spore progeny was tested by spotting (*ca.* 25 cells per sample) on YP supplemented with 2.0% D-, L-lactate. Plates were incubated at 30°C and 37°C for 5 days. All spores appeared to grow normally on lactate at 30°C.

Table 4. Yeast generation times: Pseudo-revertants.

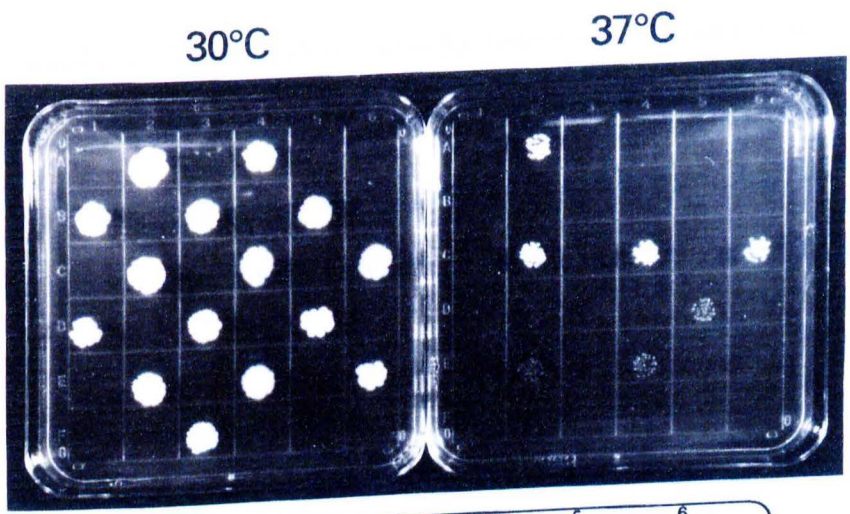
Strain	Generation time at 28°C (hours)
MB11 (*CPR3*$^+$)	4.3
MB11-3 (*cpr3*)	5.4
ED259-1 (*cpr3*)	5.2
ED260-1 (*cpr3*)	4.8
ED261-1 (*cpr3*)	4.8

All strains were cultured in synthetic minimal media containing 2.0% glucose overnight at 28°C. Cells were diluted into 50 ml liquid YP supplemented with 2.0% D-, L-lactate in 0.5L baffled flasks, to an A_{600} = 0.03. Cultures were incubated with 300 rpm shaking at 28°C. The first optical density measurement took place after eight hours, and then once every four hours. Generation times were calculated from optical density measurements of cultures from 20-24 hours after inoculation. Experiment was performed three times. Data shown are from one representative trial.

Figure 1. Isolation of yeast carrying *JEN1*, a high-copy suppressor of *CPR3* disruption.

Plasmid DNA was extracted (see Materials and Methods) from strains marked with * (denotes original isolate of the strain), and was reintroduced to ED80-2 (*cpr3::HIS3*). Two independent transformants of each plasmid in ED80-2 (*e. g.* ED249-1 and ED249-2) were then tested for their ability to grow on YP supplemented with 2.0% D-, L-lactate. Strains MH272-3c (*CPR3*), ED80-2 (*cpr3::HIS3*), ED249* (*cpr3::HIS3*, YEp13 [*LEU2*], ED249-1 (*cpr3::HIS3*, YEp13 [*LEU2*]), ED249-2 (*cpr3::HIS3*, YEp13 [*LEU2*]), ED250* (*cpr3::HIS3*, pESD34 [*LEU2*, *CPR3*]), ED250-1 (*cpr3::HIS3*, pESD34 [*LEU2*, *CPR3*]), ED250-2 (*cpr3::HIS3*, pESD34 [*LEU2*, *CPR3*]), ED250-3 (*cpr3::HIS3*, *leu2*), ED250-4 (*cpr3::HIS3*, *leu2*), ED252* (*cpr3::HIS3*, pESD33 [*LEU2*, *JEN1*]), ED252-1 (*cpr3::HIS3*, pESD33 [*LEU2*, *JEN1*]), ED252-2 (*cpr3::HIS3*, pESD33 [*LEU2*, *JEN1*]), ED252-3 (*cpr3::HIS3*, *leu2*), and ED252-4 (*cpr3::HIS3*, *leu2*) were cultured in synthetic minimal medium containing 2.0% glucose overnight at 30°C. Approximately 60 cells of each strain were spotted on YP supplemented with 2.0% D-, L-lactate. Top, plates incubated at 30°C and 37°C for seven days. Bottom, schematic diagram of strain arrangement.

Figure 1.



	1	2	3	4	5	6
A		MH272-3c (*CPR3*)		ED80-2 (*cpr3*::*HIS3*)		
B	ED249* (*cpr3*::*HIS3* + YEp13)		ED249-1 (*cpr3*::*HIS3* + YEp13)		ED249-2 (*cpr3*::*HIS3* + YEp13)	
C		ED250* (*cpr3*::*HIS3* + pYE*CPR3*)		ED250-1 (*cpr3*::*HIS3* + pYE*CPR3*)		ED250-2 (*cpr3*::*HIS3* + pYE*CPR3*)
D	ED250-3 (ED250* leu⁻)		ED250-4 (ED250* leu⁻)		ED252* (*cpr3*::*HIS3* + pYE*JEN1*)	
E		ED252-1 (*cpr3*::*HIS3* + pYE*JEN1*)		ED252-2 (*cpr3*::*HIS3* + pYE*JEN1*)		ED252-3 (ED252* leu⁻)
F			ED252-4 (ED252* leu⁻)			

Figure 2. Subcloning of pYE*JEN1* (pESD33).

Schematic representation of pESD33 fragments tested for ability to suppress the *cpr3::HIS3* phenotype at 37^{0}C. Cross-hatched boxes, insert DNA of pESD33. Solid line, YEp13 DNA. Sites for restriction endonuclease cleavage are indicated: B, BamHI; S, SphI; *, original BamHI site of YEp13, destroyed by insertion of the *JEN1* Sau3AI library fragment. 1. pESD33: YEp13 + full-length (*ca.* 8.5 kb) insert containing *JEN1*. 2. pESD39: 12 kb SphI fragment of pESD33, re-circularized without internal fragments. 3. pESD40: YEp13 + 4.4kb internal SphI fragment, from pESD33. 4. pESD41: YEp13 + 2.6kb internal SphI fragment, from pESD33. 5. pESD37: 16 kb BamHI fragment of pESD33, re-circularized without internal fragment. 6. pESD38: YEp13 + internal 3.3 kb BamHI fragment from pESD33. Fragments are not drawn to scale. Two independent isolates of each plasmid were introduced into strain ED80-2 (*cpr3::HIS3*). Leu$^+$ transformants were cultured in synthetic minimal medium containing 2.0% glucose but lacking leucine overnight at 30^{0}C. Approximately 60 cells of each transformant were spotted on plates containing YP supplemented with 2.0% D-, L-lactate, and incubated at 30^{0}C and 37^{0}C for seven days.

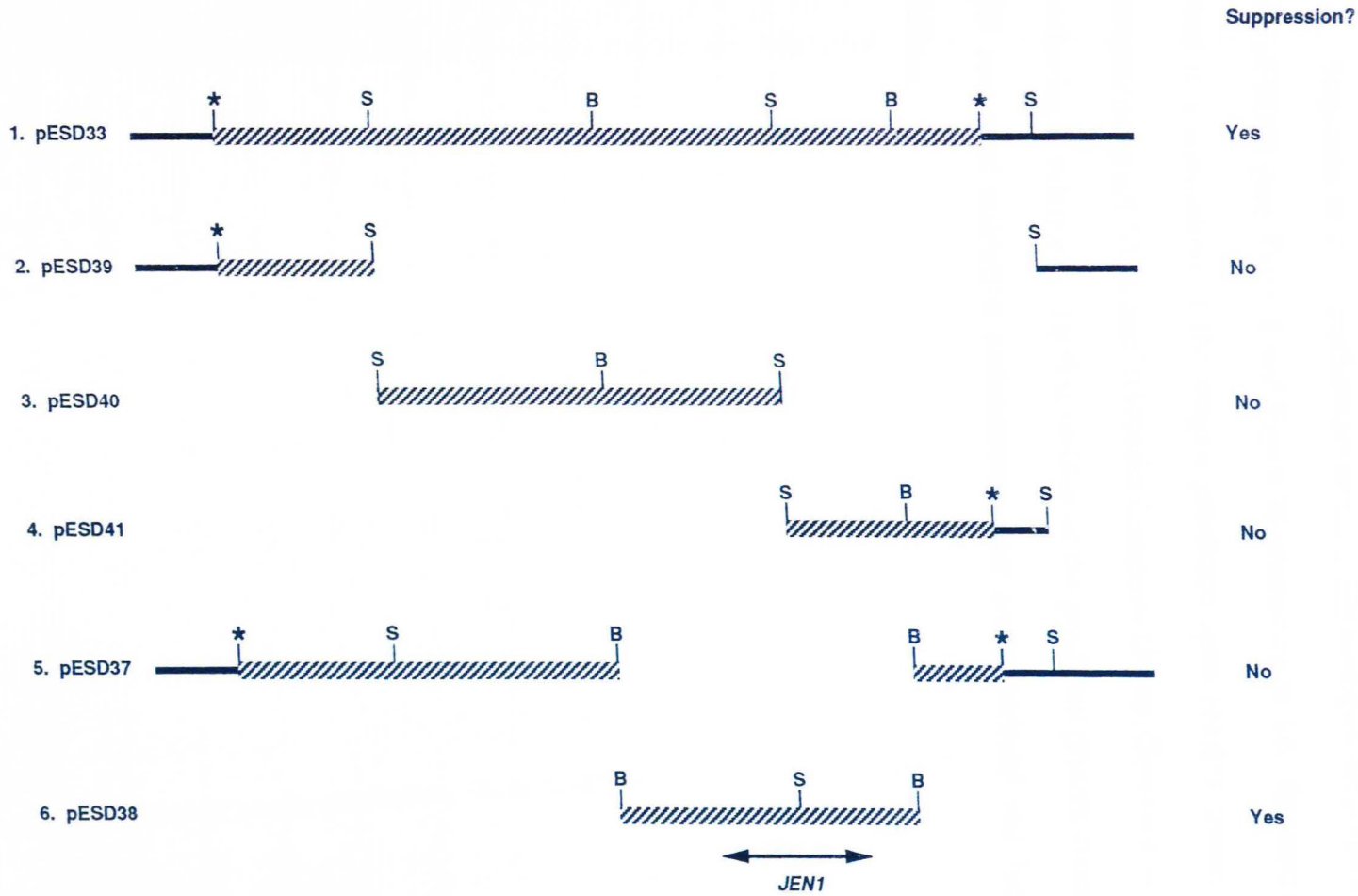

Suppression?
1. pESD33
Yes
2. pESD39
No
3. pESD40
No
4. pESD41
No
5. pESD37
No
6. pESD38
Yes
JEN1

Figure 2.

Figure 3. DNA sequence of *JEN1*.

Sequence of 2,547 nucleotides of the 3,238 base-pair suppressor fragment from pESD38 (see Table 1 and Figure 2) containing *JEN1*. Beneath the DNA strand is a translation of the longest predicted open reading frame, from the comupter program "Translate" (Genetics Computer Group; Devereux, *et al.*, 1984). Numbering is relative to the first residue of the predicted protein coding region. BglII and SphI restriction endonuclease sites are underlined and indicated in boldface.

Figure 3.

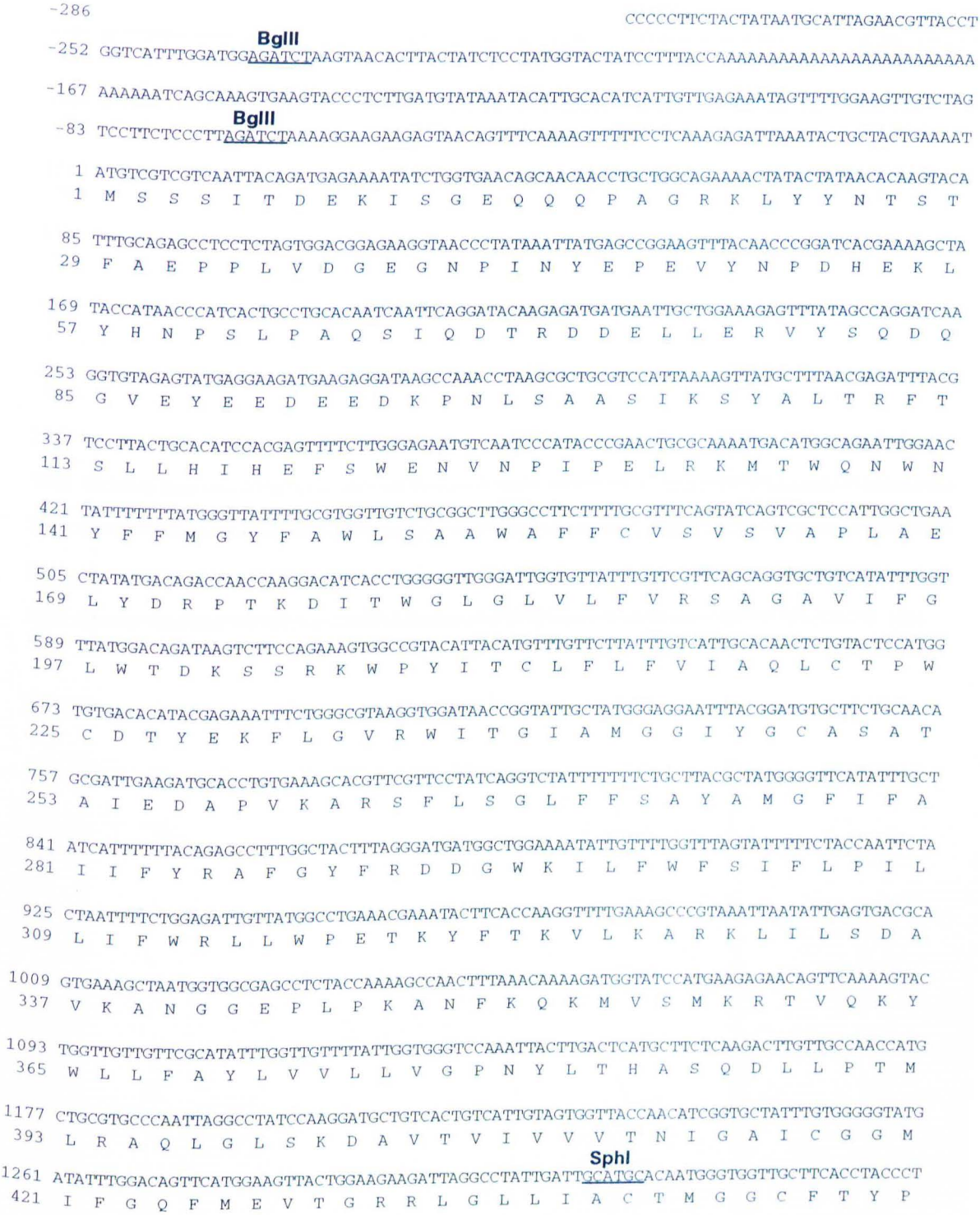

-286
CCCCCTTCTACTATAATGCATTAGAACGTTACCT
BglII
-252 GGTCATTTGGATGGAGATCTAAGTAACACTTACTATCTCCTATGGTACTATCCTTTACCAAAAAAAAAAAAAAAAAAAAAAA
-167 AAAAAATCAGCAAAGTGAAGTACCCTCTTGATGTATAAATACATTGCACATCATTGTTGAGAAATAGTTTTGGAAGTTGTCTAG
BglII
-83 TCCTTCTCCCTTAGATCTAAAAGGAAGAAGAGTAACAGTTTCAAAAGTTTTTCCTCAAAGAGATTAAATACTGCTACTGAAAAT
1 ATGTCGTCGTCAATTACAGATGAGAAAATATCTGGTGAACAGCAACAACCTGCTGGCAGAAAACTATACTATAACACAAGTACA
1 M S S S I T D E K I S G E Q Q Q P A G R K L Y Y N T S T
85 TTTGCAGAGCCTCCTCTAGTGGACGGAGAAGGTAACCCTATAAATTATGAGCCGGAAGTTTACAACCCGGATCACGAAAAGCTA
29 F A E P P L V D G E G N P I N Y E P E V Y N P D H E K L
169 TACCATAACCCATCACTGCCTGCACAATCAATTCAGGATACAAGAGATGATGAATTGCTGGAAAGAGTTTATAGCCAGGATCAA
57 Y H N P S L P A Q S I Q D T R D D E L L E R V Y S Q D Q
253 GGTGTAGAGTATGAGGAAGATGAAGAGGATAAGCCAAACCTAAGCGCTGCGTCCATTAAAAGTTATGCTTTAACGAGATTTACG
85 G V E Y E E D E E D K P N L S A A S I K S Y A L T R F T
337 TCCTTACTGCACATCCACGAGTTTTCTTGGGAGAATGTCAATCCCATACCCGAACTGCGCAAAATGACATGGCAGAATTGGAAC
113 S L L H I H E F S W E N V N P I P E L R K M T W Q N W N
421 TATTTTTTTATGGGTTATTTTGCGTGGTTGTCTGCGGCTTGGGCCTTCTTTTGCGTTTCAGTATCAGTCGCTCCATTGGCTGAA
141 Y F F M G Y F A W L S A A W A F F C V S V S V A P L A E
505 CTATATGACAGACCAACCAAGGACATCACCTGGGGGTTGGGATTGGTGTTATTTGTTCGTTCAGCAGGTGCTGTCATATTTGGT
169 L Y D R P T K D I T W G L G L V L F V R S A G A V I F G
589 TTATGGACAGATAAGTCTTCCAGAAAGTGGCCGTACATTACATGTTTGTTCTTATTTGTCATTGCACAACTCTGTACTCCATGG
197 L W T D K S S R K W P Y I T C L F L F V I A Q L C T P W
673 TGTGACACATACGAGAAATTTCTGGGCGTAAGGTGGATAACCGGTATTGCTATGGGAGGAATTTACGGATGTGCTTCTGCAACA
225 C D T Y E K F L G V R W I T G I A M G G I Y G C A S A T
757 GCGATTGAAGATGCACCTGTGAAAGCACGTTCGTTCCTATCAGGTCTATTTTTTTTCTGCTTACGCTATGGGGTTCATATTTGCT
253 A I E D A P V K A R S F L S G L F F S A Y A M G F I F A
841 ATCATTTTTTTACAGAGCCTTTGGCTACTTTAGGGATGATGGCTGGAAAATATTGTTTTTGGTTTAGTATTTTTCTACCAATTCTA
281 I I F Y R A F G Y F R D D G W K I L F W F S I F L P I L
925 CTAATTTTCTGGAGATTGTTATGGCCTGAAACGAAATACTTCACCAAGGTTTTTGAAAGCCCGTAAATTAATATTGAGTGACGCA
309 L I F W R L L W P E T K Y F T K V L K A R K L I L S D A
1009 GTGAAAGCTAATGGTGGCGAGCCTCTACCAAAAGCCAACTTTAAACAAAAGATGGTATCCATGAAGAGAACAGTTCAAAAGTAC
337 V K A N G G E P L P K A N F K Q K M V S M K R T V Q K Y
1093 TGGTTGTTGTTCGCATATTTGGTTGTTTTATTGGTGGGTCCAAATTACTTGACTCATGCTTCTCAAGACTTGTTGCCAACCATG
365 W L L F A Y L V V L L V G P N Y L T H A S Q D L L P T M
1177 CTGCGTGCCCAATTAGGCCTATCCAAGGATGCTGTCACTGTCATTGTAGTGGTTACCAACATCGGTGCTATTTGTGGGGGTATG
393 L R A Q L G L S K D A V T V I V V V T N I G A I C G G M
SphI
1261 ATATTTGGACAGTTCATGGAAGTTACTGGAAGAAGATTAGGCCTATTGATTGCATGCACAATGGGTGGTTGCTTCACCTACCCT
421 I F G Q F M E V T G R R L G L L I A C T M G G C F T Y P

Figure 3. (continued)

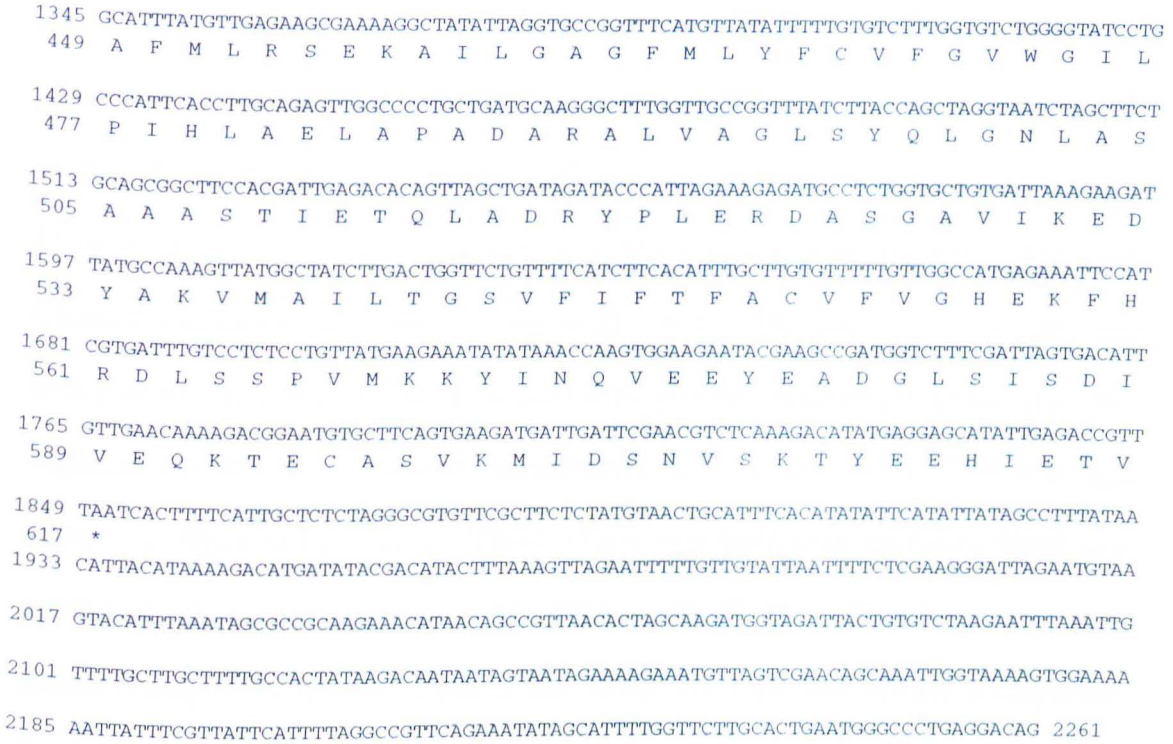


Figure 4. The predicted *JEN1* protein is similar to a family of transporters.

The Genetics Computer Group program "Bestfit" was used to generate alignments between Jen1 and ProP (*E. coli* proline/betaine transporter; Culham, *et al.*, 1993), between Jen1 and Bmr (*C. albicans* methotrexate resistance protein; Fling, *et al.*, 1991), and between Jen1 and Mct (lactate/pyruvate transporter from Chinese hamster ovary cells; Garcia, *et al.*, 1994). Mct was originally identified as a protein that, due to a phenylalanine-to-cysteine transversion at position 360, acquires the ability to transport mevalonate (Kim, *et al.*, 1992). The wild-type protein is shown in this figure. These alignments were then manually fitted to a published alignment (Culham, *et al.*, 1993) of ProP, Cit (*Klebsiella pneumoniae* citrate transporter; van der Rest, *et al.*, 1990), Ciu1 (*E. coli* citrate transporter; Susatsu, *et al.*, 1985), and Kgtp (*E. coli* α-ketoglutarate transporter; Seol and Shatkin, 1991). Shading indicates Jen1 residues in common with at least one additional protein. Boxes denote residues common among other sequences but absent in Jen1.

Figure 4.

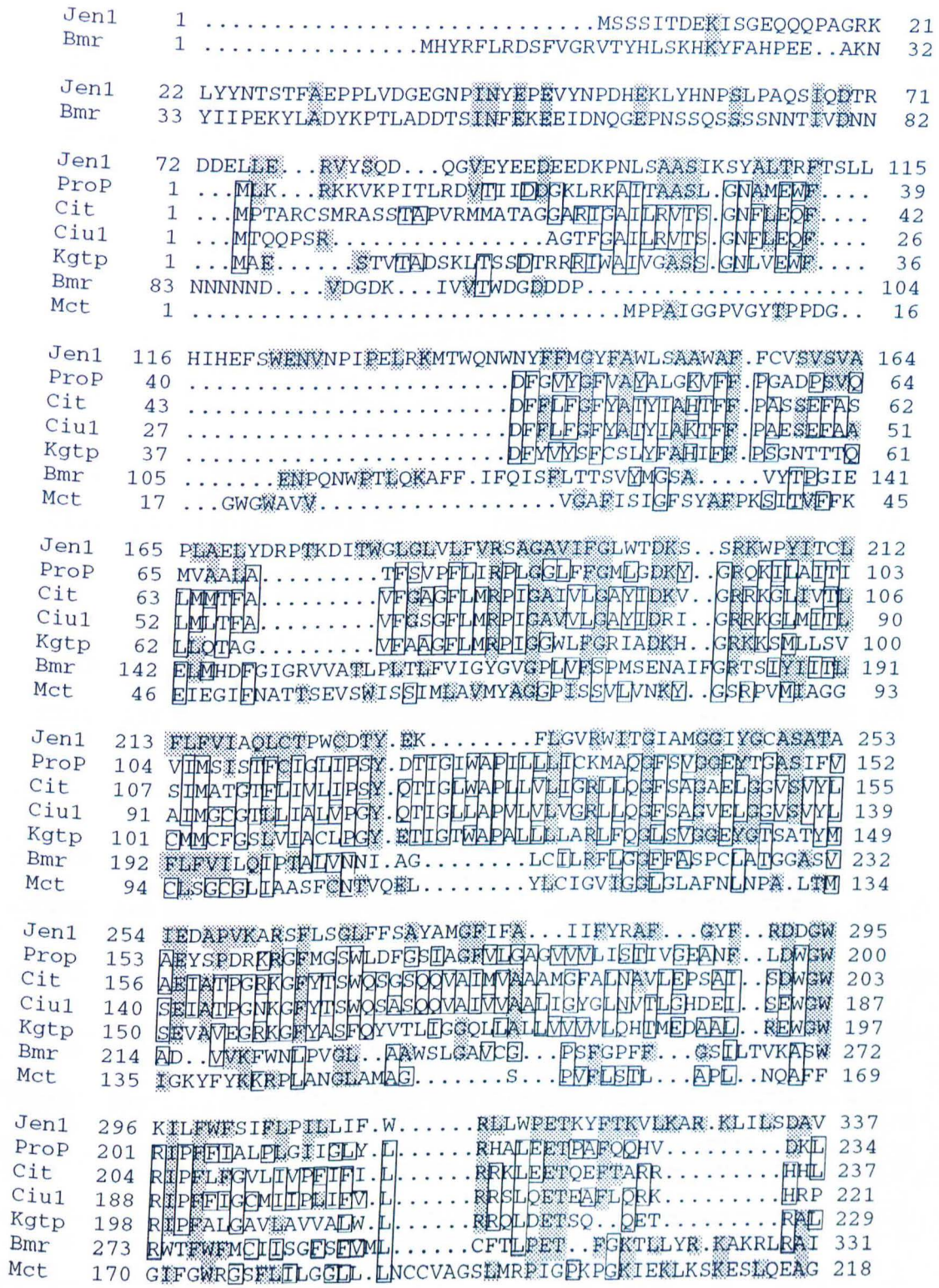

Jen1 1 ..... MSSSITDEKISGEQQQPAGRK 21
Bmr 1 ..... MHYRFLRDSFVGRVTYHLSKHKYFAHPEE..AKN 32
Jen1 22 LYYNTSTFAEPPLVDGEGNPINYEPEVYNPDHEKLYHNPSLPAQSIQDTR 71
Bmr 33 YIIPEKYLADYKPTLADDTSINFEKEEIDNQGEPNSSQSSSSNNTIVDNN 82
Jen1 72 DDELLE...RVYSQD...QGVEYEEDEEDKPNLSAASIKSYALTRFTSLL 115
ProP 1 ...MLK...RKKVKPITLRDVTIIDDGKLRKAITAASL.GNAMEWF.... 39
Cit 1 ...MPTARCSMRASSTAPVRMMATAGGARIGAILRVTS.GNFLEQF.... 42
Ciu1 1 ...MTQQPSR..... AGTFGAILRVTS.GNFLEQF.... 26
Kgtp 1 ...MAE..... STVTADSKLTSSDTRRRIWAIVGASS.GNLVEWF.... 36
Bmr 83 NNNNND....VDGDK...IVVTWDGDDDP..... 104
Mct 1 ..... MPPAIGGPVGYTPPDG.. 16
Jen1 116 HIHEFSWENVNPIPELRKMTWQNWNYFFMGYFAWLSAAWAF.FCVSVSVA 164
ProP 40 ..... DFGVVGFVAVALGKVFF.PGADPSVQ 64
Cit 43 ..... DFFLFGFYAIYIAHIFF.PASSEFAS 62
Ciu1 27 ..... DFFLFGFYALYIAKIFF.PAESEFAA 51
Kgtp 37 ..... DFYVVSFCSLYFAHIFF.PSGNTTTQ 61
Bmr 105 .....ENPQNWPTLQKAFF.IFQISFLTTSVYMGSA.....VYTPGIE 141
Mct 17 ...GWGWAVV..... VGAFISIGFSYAFPKSITVFFK 45
Jen1 165 PLAELYDRPTKDITWGLGLVLFVRSAGAVIFGLWTDKS..SRKWPYITCL 212
ProP 65 MVAALA..... TFSVPFFLIRPLGGLFFGMLGDKY..GRQKILAITI 103
Cit 63 LMMTFA..... VFGAGFLMRPIGAIVLGAYIDKV..GRRKGLIVTL 106
Ciu1 52 LMLTFA..... VFGSGFLMRPIGAVVLGAYIDRI..GRRKGLMITL 90
Kgtp 62 LLQTAG..... VFAAGFLMRPIGGWLFGRIADKH..GRKKSMLLSV 100
Bmr 142 ELMHDFGIGRVVATLPLTLFVIGYGVGPLVFSPMSENAIFGRTSIYIITL 191
Mct 46 EIEGIFNATTSEVSWISSIMLAVMYAGGPISSVLVNKY..GSRPVMIAGG 93
Jen1 213 FLFVIAQLCTPWCDTY.EK..... FLGVRWITGIAMGGIYGCASATA 253
ProP 104 VIMSISIFCIGLIPSY.DTIGIWAPILLLLICKMAQGFSVGGEYTGASIFV 152
Cit 107 SIMATGTFLIMLIPSY.QTIGLWAPLLVLIGRLLQGFSAGAELGGVSVYL 155
Ciu1 91 AIMGCGTILIALVPGY.QTIGLLAPVLVLVGRLLQGFSAGVELGGVSVYL 139
Kgtp 101 CMMCFGSLVIACLPGY.ETIGTWAPALLLLARLFQGLSVGGEYGTSATYM 149
Bmr 192 FLFVILQIPTALVNNI.AG..... LCILRFLCGFFASPCLAIGGASV 232
Mct 94 CLSGCGLIAASFCNTVQEL..... YLCIGVIGGLGLAFNLNPA.LTM 134
Jen1 254 IEDAPVKARSFLSGLFFSAYAMGFIFA...IIFYRAF...GYF..RDDGW 295
Prop 153 AEYSPDRKRGFMGSWLDFGSIAGFVLGAGVVVLISIIVGFANF..LDWGW 200
Cit 156 AEIAPPGRKGFYTSWQSGSQQVAIMVAAAMGFALNAVLEPSAI..SDWGW 203
Ciu1 140 SEIAPPGNKGFYTSWQSASQQVAIVVAALIGYGLNVTLGHDEI..SEWGW 187
Kgtp 150 SEVAVEGRKGFYASFQYVTLIGGQLLALLVVVVLQHIMEDAAL..REWGW 197
Bmr 214 AD..VVKFWNLPVGL..AAWSLGAVCG...PSFGPFF...GSILTVKASW 272
Mct 135 IGKYFYKKRPLANGLAMAG.....S...PVFLSTL...APL..NQAFF 169
Jen1 296 KILFWFSIFLPILLIF.W..... RLLWPETKYFTKVLKAR.KLILSDAV 337
ProP 201 RIPFFIALPLGIIGLY.L..... RHALEEIPAFQQHV..... DKL 234
Cit 204 RIPFLFGVLIVPFIFI.L..... RRKLEETQEFTARR..... HHL 237
Ciu1 188 RIPFFIGGMIIPLIFV.L..... RRSLQETEAFLQRK..... HRP 221
Kgtp 198 RIPFALGAVLAVVALW.L..... RRQLDETSQ..QET..... RAL 229
Bmr 273 RWTFWFMCIISGFSFVML..... CFTLPET..FGKTLLYR.KAKRLRAI 331
Mct 170 GIFGWRGSFLILGGLL.LNCCVAGSLMRPIGPKPGKIEKLKSKESLQEAG 218

Figure 4. (continued)

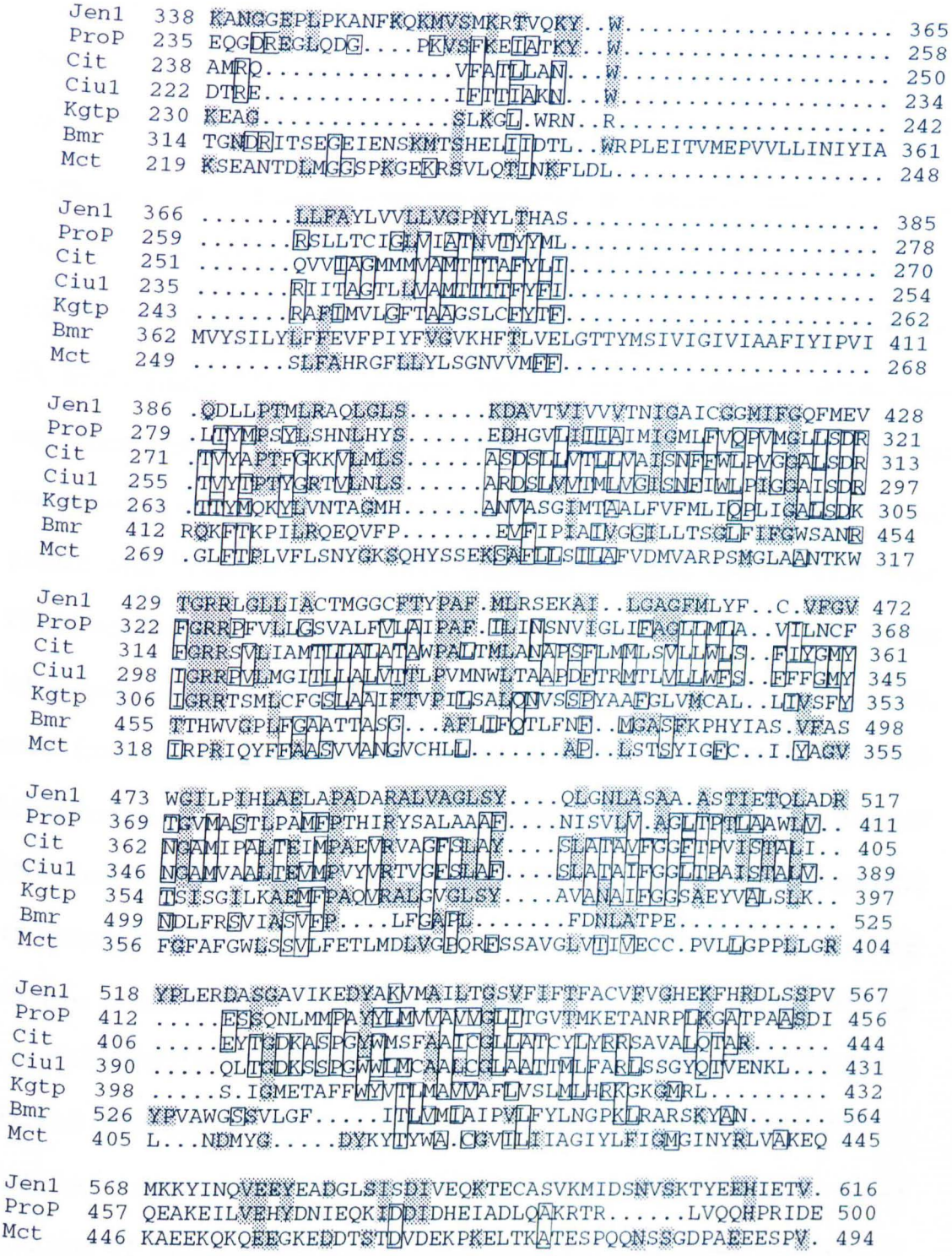


Figure 5. Deletion analysis of *JEN1*.

A. Schematic representation of *JEN1* alleles. Top, wild-type (*JEN1*$^+$). Bottom, *jen1* deletion allele. Patterned boxes represent protein coding regions of *JEN1* and *URA3*. Flanking solid lines represent non-coding regions. Sites for restriction endonuclease cleavage are indicated: B, BamHI; G, BglII; S, SphI. B. Northern blot analysis of *JEN1* mRNA expression in strains MH272-3c (*JEN1*) and ED282-1 (*jen1::URA3*). Carbon sources used to supplement YP are indicated: D, 10.0% glucose; G, 3.0% glycerol; and L, 2.0% D-, L-lactate. RNAs from equivalent numbers of cells were loaded in duplicate on a 1.0% agarose gel containing 3.0% (w/v) formaldehyde, transferred to a nylon membrane, and probed with ^{32}P-labeled *JEN1* DNA fragments indicated beneath each filter. Filters were exposed to Kodak XAR film with one DuPont Cronex Lightning Plus intensifying screen at -80^{0}C for 2 days. Molecular weight markers, in kilobases, were from electrophoresis of a mixture of standards (Bethesda Research Laboratories), probed separately with ^{32}P-labeled bacteriophage λ DNA. Blots of RNA from MH272-3c (*JEN1*) and ED282-2 (*jen1::URA3*) performed in parallel are indistinguishable from this one (not shown). C. Growth of yeast carrying wild-type or deletion alleles of *JEN1* on 0.1M L-lactate medium. Strains MH272-3c (*CPR3, JEN1*), ED80-2 (*cpr3::HIS3, JEN1*), ED282-1 (*CPR3, jen1::URA3*), and ED282-2 (*CPR3, jen1::URA3*) were cultured in liquid YPD overnight at 30^{0}C to saturation. Approximately 60 cells of each strain were spotted on plates containing YP supplemented with 100mM L-lactate. Top, plates incubated at

Figure 5. (continued)

30^0C and 37^0C for six days. Bottom, schematic diagram of strain arrangement.

Figure 5A.

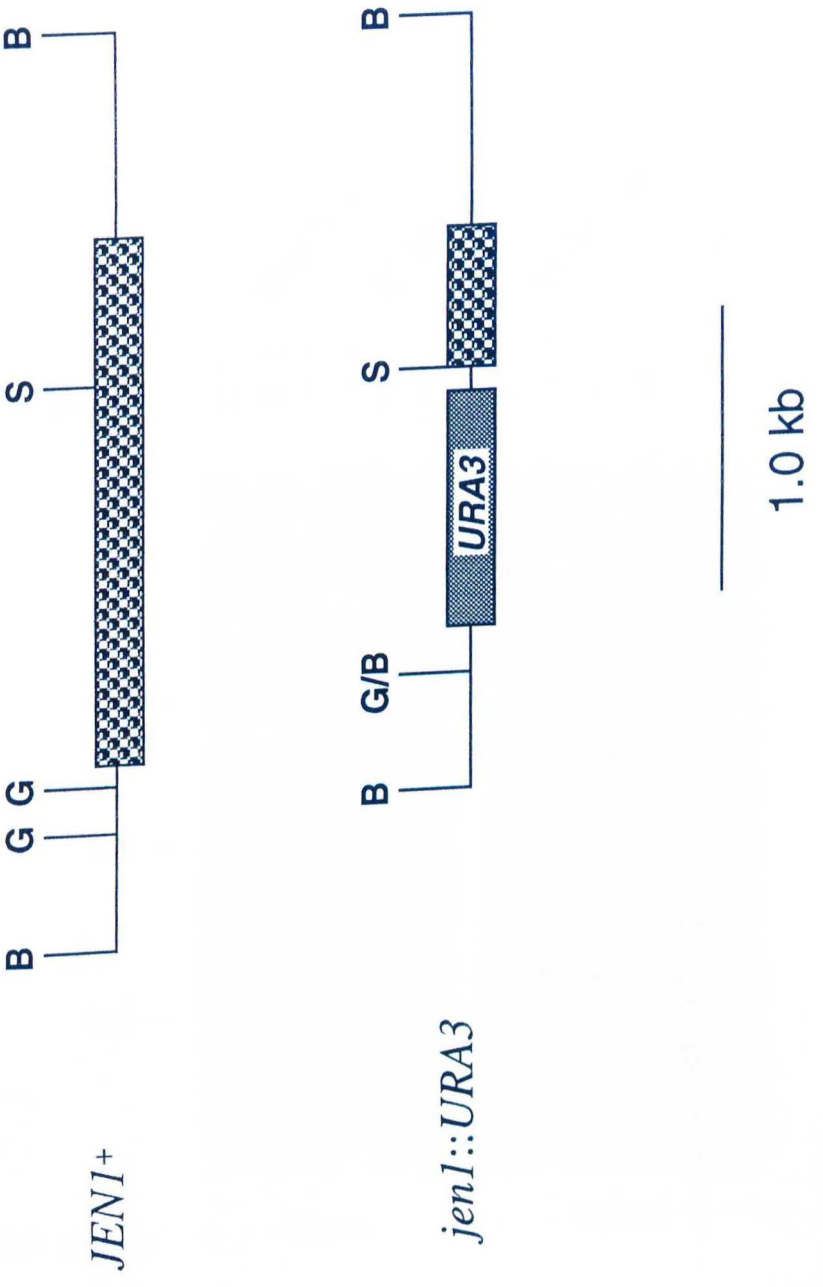


Figure 5B.

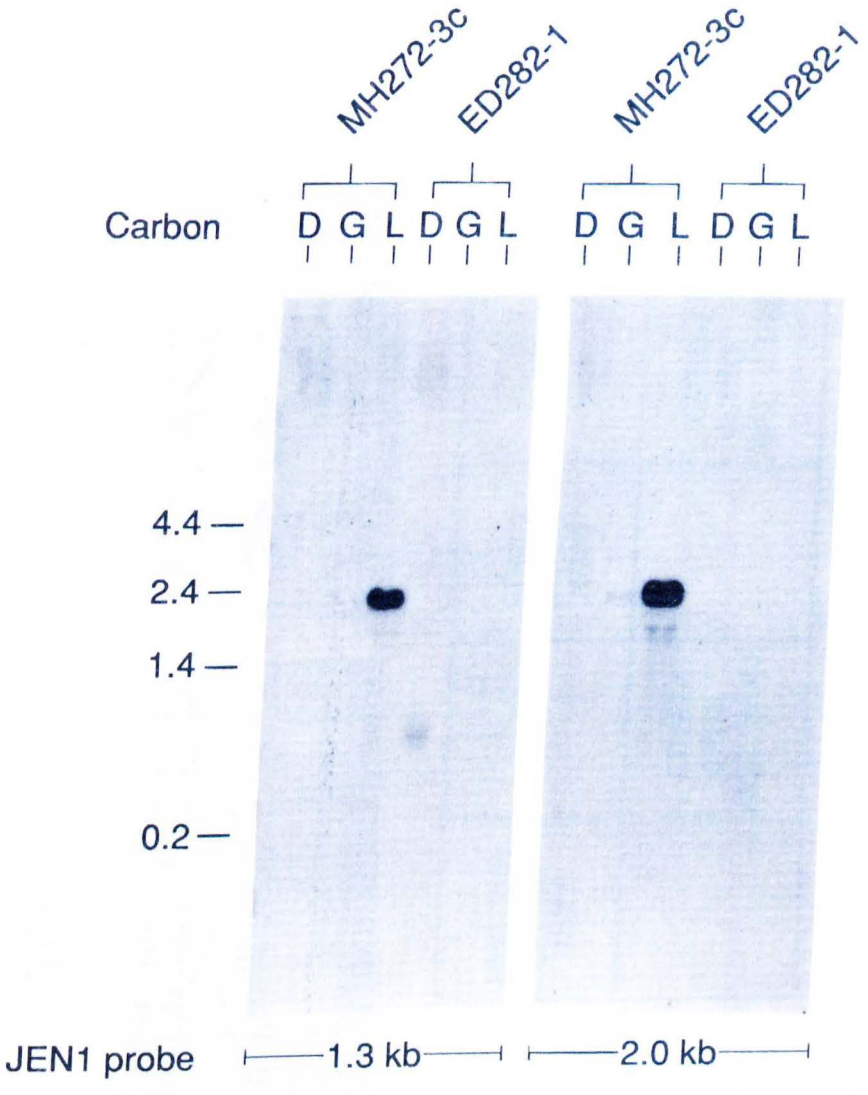

MH272-3c
ED282-1
MH272-3c
ED282-1
Carbon
D G L D G L D G L D G L
4.4
2.4
1.4
0.2
JEN1 probe
1.3 kb
2.0 kb

Figure 5C.

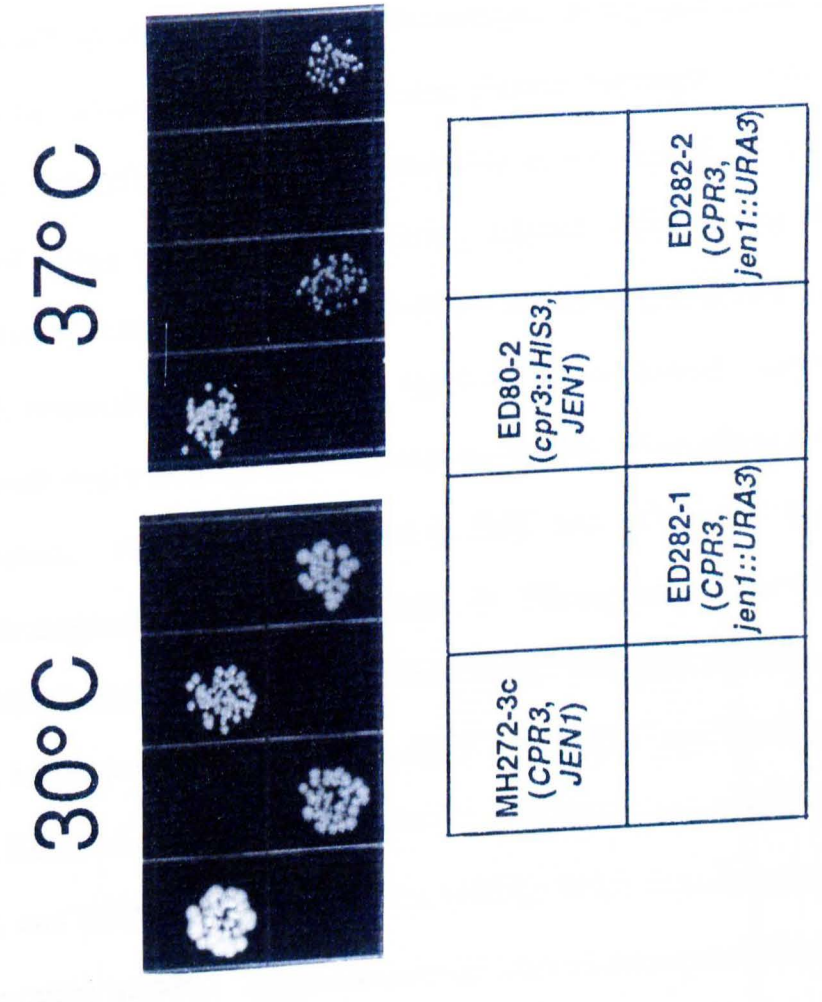

30°C
37°C
MH272-3c (CPR3, JEN1)
ED282-1 (CPR3, jen1::URA3)
ED80-2 (cpr3::HIS3, JEN1)
ED282-2 (CPR3, jen1::URA3)

Figure 6. Isolation of pseudo-revertants.

A. Pseudo-revertants of MB11-3 (*cpr3::HIS3*) carry dominant suppressor mutations. Strains MB11 (*CPR3*), MB11-3 (*cpr3::HIS3*), ED179 (*CPR3/CPR3*), ED180 (*CPR3/cpr3::HIS3*), ED181 (*cpr3::HIS3/cpr3::HIS3*), ED259-1 (*cpr3::HIS3*), ED260-1 (*cpr3::HIS3*), ED261-1 (*cpr3::HIS3*), ED262 (*cpr3::HIS3/cpr3::HIS3*), ED263 (*cpr3::HIS3/cpr3::HIS3*), and ED264 (*cpr3::HIS3/cpr3::HIS3*) were cultured in synthetic minimal medium containing glucose overnight at 30^0C. ED259-1, ED260-1, and ED261-1 arose spontaneously at the rate of *ca.* 2 X 10^{-5} in the course of other experiments. ED181, ED262, ED263, and ED264 were constructed by mating ED128-1 (*cpr3::HIS3*; not shown), ED259-1, ED260-1, and ED261-1, respectively, with ED134-1 (*cpr3::HIS3*; not shown). Approximately 25 cells of each strain were spotted on plates containing YP supplemented with 2.0% D-, L-lactate. Top, plates incubated at 30^0C and 37^0C for 5 days. Bottom, schematic diagram of strain arrangement. B. The suppressor in ED261 is not an allele of *JEN1*. Strains MB11 (*CPR3, JEN1, JEN2*$^+$), ED280-1 (*CPR3, jen1::URA3, JEN2*$^+$), MB11-3 (*cpr3::HIS3, JEN1, JEN2*$^+$), ED281-1 (*cpr3::HIS3, jen1::URA3, JEN2*$^+$), ED261-2 (*cpr3::HIS3, JEN1, JEN2-1*), ED287-1 (*cpr3::HIS3, jen1::URA3, JEN2-1*), and ED287-2 (*cpr3::HIS3, jen1::URA3, JEN2-1*) were cultured in liquid YPD overnight at 30^0C. Approximately 25 cells of each strain were spotted on plates containing YP supplemented with 2.0% D-, L-lactate. Top, plates incubated at 30^0C and 37^0C for five days. Bottom, schematic diagram of strain arrangement.

Figure 6A.

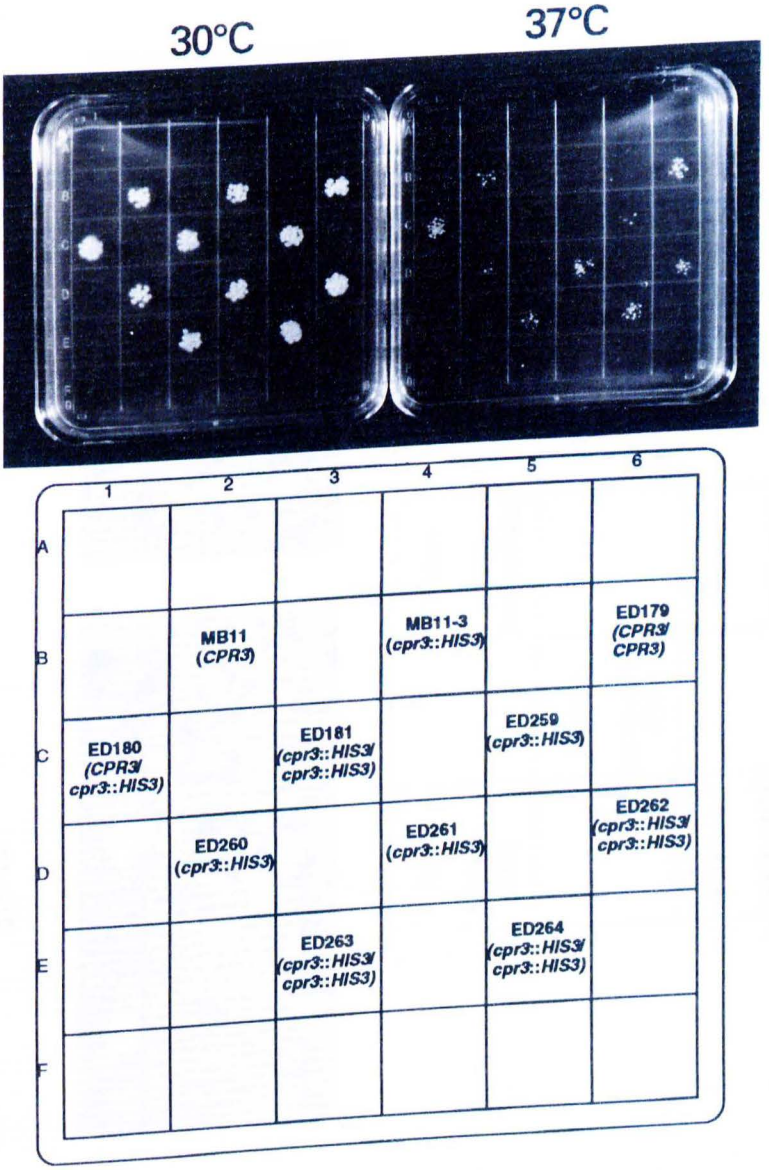
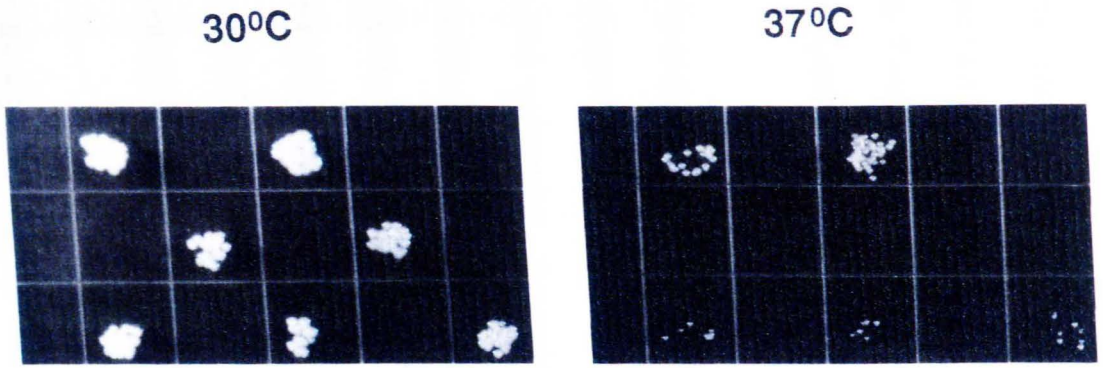

30°C
37°C
1
2
3
4
5
6
A
B
C
D
E
F
MB11 (CPR3)
MB11-3 (cpr3::HIS3)
ED179 (CPR3/ CPR3)
ED180 (CPR3/ cpr3::HIS3)
ED181 (cpr3::HIS3/ cpr3::HIS3)
ED259 (cpr3::HIS3)
ED260 (cpr3::HIS3)
ED261 (cpr3::HIS3)
ED262 (cpr3::HIS3/ cpr3::HIS3)
ED263 (cpr3::HIS3/ cpr3::HIS3)
ED264 (cpr3::HIS3/ cpr3::HIS3)

Figure 6B.



MB11 (*CPR3*, *JEN1*)		ED280-1 (*CPR3*, *jen1::URA3*)		
	MB11-3 (*cpr3::HIS3*, *JEN1*)		ED281-1 (*cpr3::HIS3*, *jen1::URA3*)	
ED261-2 (*cpr3::HIS3*, *JEN1*)		ED287-1 (*cpr3::HIS3*, *jen1::URA3*)		ED287-2 (*cpr3::HIS3*, *jen1::URA3*)

Chapter 5. General Discussion

Section A: Introduction

The principal question that I have addressed in this work is: What are the functions of the cyclophilins? Previous work, outlined in chapter 1, suggests two possible *in vivo* roles for cyclophilins: Regulation of signal transduction pathways, such as in T-cell activation, or protein folding. To date, the evidence for the two predicted roles has not been integrated into a model of cyclophilin function.

Role of cyclophilins in signal transduction. Cyclophilins are required for the immunosuppressive and cytotoxic activities of cyclosporin A (CsA). CsA forms a complex with cyclophilin, which probably inhibits the phosphatase calcineurin, in T-cell activation (Clipstone and Crabtree, 1992; O'Keefe, *et al.*, 1992), or an as yet unidentified factor essential for viability in *N. crassa* and CsA-sensitive mutants of *S. cerevisiae* (Tropschug, *et al.*, 1989). These findings suggest that cyclophilins play roles in signal transduction pathways. It is not yet known if calcineurin participates in the cytotoxic activity of CsA (see section C of this chapter).

Evidence for a protein folding role for cyclophilins. Cyclophilins have PPIase, or proline isomerase, activity *in vitro* (Fischer, *et al.*, 1989). Because proline isomerization is a rate limiting step of protein folding *in vitro* (see review in chapter 1), the physiological functions of cyclophilins might be to catalyze protein

folding. To date, the best evidence supporting a protein folding model for cyclophilins comes from the *D. melanogaster ninaA* gene product, which is necessary for the efficient processing of Rh1 rhodopsin in the secretory pathway of photoreceptor cells. However, there is still no evidence that rhodopsin is a direct biochemical target of the *ninaA* gene product.

Chemical mutagenesis of *D. melanogaster* revealed that two point mutations in the *ninaA* gene, leading to Rh1 rhodopsin deficiency in flies, also abolish PPIase activity in a rat cyclophilin isoform (Ondek, *et al.*, 1992). This result suggests that cyclophilin PPIase activity is relevant for protein folding *in vivo*. However, it is still not known if the *ninaA* gene product has PPIase activity. It is possible that these mutations have other deleterious effects on the *ninaA* gene product, not related to PPIase activity. These mutations are in one of the most highly evolutionarily conserved regions of cyclophilins. As discussed in chapter 1, certain mutations in highly conserved residues of human cyclophilin A cause a much greater loss of CsA-binding activity compared with PPIase activity (Liu, *et al.*, 1991b), suggesting that evolutionary pressure on cyclophilin extends beyond maintenance of PPIase activity to include structures necessary for CsA binding. These structures have been maintained, of course, for some function other than CsA binding, but we do not know what that function is. Any mutations knocking out PPIase activity could also be abolishing this other cyclophilin function. Thus, the indirect nature of this result makes analysis of the physiological relevance of PPIase activity difficult.

The normal physiological roles of cyclophilins are enigmatic, as some

evidence suggests a protein folding role, whereas other evidence suggests that cyclophilins might be involved in the PPIase-independent regulation of calcineurin. How can this apparent paradox be resolved? One can speculate that the *in vitro* PPIase activity of cyclophilins is an artifact. Alternatively, since CsA is not a normal physiological component of eukaryotic cells, it is possible that the biological activities of CsA are unrelated to the normal functions of cyclophilins. This latter possibility is illustrated by comparisons of known mechanisms of other immunosuppressants.

The FKBPs (FK-506 binding proteins) share no significant amino acid similarity with cyclophilins, yet show remarkable biological similarity (Liu, *et al.*, 1991). FKBPs have PPIase activity *in vitro*, which is inhibited by the immunosuppressant ligand, FK-506 (Harding, *et al.*, 1989). FK-506 also inhibits the calcineurin-dependent step in T-cell activation of lymphokine gene transcription, and inhibition of FKBP PPIase is not a factor in calcineurin inactivation (Flanagan, *et al.*, 1991; Bierer, *et al.*, 1990a). FKBP also plays a role in the rapamycin-sensitivity of T-lymphocyte activation. However, while rapamycin also binds FKBP and inhibits its PPIase activity, it instead inhibits the signal transduction pathway involved in the response of T-cells to interleukin 2 (Sigal and Dumont, 1992). Rapamycin and FK-506 antagonize each others' effects, suggesting that they bind the same receptor, but inhibit different signal transduction pathways (Bierer, *et al.*, 1990b).

It is curious that two proteins unrelated in amino acid sequence, cyclophilin and FKBP, mediate inhibition of the same signal transduction pathway in T-

lymphocytes. If the actions of these immunosuppressant ligands truly mimic the natural functions of the immunophilins, how can we understand the observation that FKBP also mediates inhibition of a different signal transduction pathway, when it binds in a complex with rapamycin?

One possible interpretation of these data is that the biological actions of CsA do not reflect the normal functions of cyclophilins. As discussed in chapter 1, the fact that CsA and FK-506 have very similar biological activities could be due to formation of complexes with similar three-dimensional structures (Schreiber, 1992). The cyclophilin structure is maintained by a selection for some physiological function it possesses, but this structure is "hijacked" by cyclosporin A. The complex of cyclophilin and CsA, in this model, can inhibit calcineurin, although in the absence of cyclosporin A, cyclophilin and calcineurin are not functionally related. Perhaps the microorganisms that produce these immunosuppressants have evolved efficient means of co-opting conserved protein structures to effect cytotoxicity.

Thus, while considerable progress has been made in elucidating the mechanism of immunosuppressant drug action, it is difficult to reconcile this information with the known PPIase activity of cyclophilins into a model for cyclophilin function. In order to address the problem of cyclophilin function, it is important to identify natural cellular factors that interact with cyclophilins, be they substrates, or endogenous ligands. A genetic system, whereby cyclophilin mutations could be constructed and studied, seemed the most appropriate means by which to study cyclophilin function. But, due to the large number of cyclophilin

genes (Danielson, *et al.*, 1988), it would be impractical to take such an approach in a mammalian system. For this reason, I chose the yeast, *Saccharomyces cerevisiae*, as the experimental system.

Section B: Summary of Results.

The *S. cerevisiae CPR1* and *CPR3* genes were isolated by cross-hybridization with 1B15, a rat brain cyclophilin cDNA (see chapter 2). The predicted proteins of *CPR1* and *CPR3* share 70% amino acid identity; Cpr3 shares 62% identity with the 1B15 predicted protein. I determined by insertional mutagenesis that *CPR1*, *CPR2*, and *CPR3*, in any combination, are not essential for viability on rich or minimal media (chapter 2; McLaughlin, *et al.*, 1992). Analysis of crude extracts indicates that *CPR1* encodes the major CsA binding protein in yeast (chapter 3).

Rabbit polyclonal antisera raised against two peptides of the *CPR3* gene product, Cpr3, recognize only one yeast cyclophilin (chapter 3). Western blots probed with these antisera show that *cpr3::HIS3* strains do not produce detectable levels of Cpr3. Therefore, *cpr3::HIS3* is a null allele of *CPR3* (chapter 3). These antisera recognize both full-length and truncated versions of Cpr3 expressed in *E. coli* (chapter 3).

Cpr3 expressed in *E. coli* binds CsA (chapter 3), consistent with results of others (McLaughlin, *et al.*, 1992), and is localized in the mitochondrial matrix (chapter 3). Mitochondrial localization is consistent with sequence prediction, and restricts analysis of the phenotype of *cpr3::HIS3* mutants (see below).

S. cerevisiae lacking functional alleles of *CPR3* are unable to grow on rich medium supplemented with lactic acid at 37^0C (chapter 2). This phenotype is not lethal; cell growth can be partially restored by increasing the lactate concentration in the medium, plating cells at high density, or by returning the plates to lower

temperatures after up to seven days at the restrictive temperature (unpublished observations). So, the observed phenotype of *cpr3::HIS3* mutants is probably one of stasis. Moreover, *cpr3::HIS3* strains exhibit a slow growth phenotype on non-fermentable carbon sources at 30^0C, the optimal growth temperature for *S. cerevisiae* (chapter 3). Therefore, the physiological role of Cpr3 is not limited to protecting cells from temperature stress.

What is the biochemical nature of the phenotype resulting from *CPR3* inactivation? While *cpr3::HIS3* mutants are also hampered in their ability to grow on two other non-fermentable carbon sources, pyruvate and glycerol, at 37^0C (chapter 2), they are still able to grow under these conditions. In addition, the enzyme flavocytochrome b_2, a mitochondrial lactate dehydrogenase, was reported to be essential for growth on lactate (Guiard, 1985). This knowledge initially suggested that the steps specific for lactate metabolism might be especially sensitive to the loss of Cpr3.

Past work on cyclophilins suggested one of two general possibilities to explain the phenotype of *cpr3::HIS3* mutants. First, cyclophilins might play natural roles in regulating signal transduction pathways. Expression of nuclear cytochrome genes, including *CYC1*, a gene encoding iso-1-cytochrome c, is governed by a signal transduction pathway from mitochondria to the nucleus (reviewed in Forsburg and Guarente, 1989). Not all of the biochemical components of this pathway are known. Expression of *CYB2*, the gene for the mitochondrial lactate dehydrogenase flavocytochrome b_2, might be regulated in a similar fashion (Lodi and Guiard, 1991). Therefore, Cpr3 might be involved in regulating a pathway of

signalling in yeast.

Second, the PPIase activity of cyclophilins suggests that they might be necessary for the folding of some proteins *in vivo*. Flavocytochrome b_2 was an attractive initial candidate target for Cpr3 function, so its activity in *cpr3::HIS3* mutants was examined directly.

Role of Cpr3 in signal transduction. At 30^0C, the average generation time of a *cpr3::HIS3* mutant is consistently more than 50% longer than wild-type (chapter 3). During induction at 30^0C for 2 hours or during incubation for 22 hours, the *cpr3::HIS3* mutation does not cause a signigficant decrease in the expression of *CYB2* or *CYC1* (chapter 3). At 37^0C, overall mRNA production is decreased in both wild-type and a *cpr3::HIS3* mutant, due to a secondary effect of the temperature stress. These results demonstrate that Cpr3 does not function in the signal transduction pathway regulating cytochrome gene expression.

Role of Cpr3 in flavocytochrome b_2 maturation. Flavocytochrome b_2 activity is slightly reduced in a *cpr3::HIS3* mutant incubated at 30^0C, and much more dramatically reduced at 37^0C (chapter 3). However, strains harboring null mutations of *CYB2* grow under conditions that prohibit growth of *cpr3::HIS3* mutants (chapter 3). Thus, an absence of flavocytochrome b_2 is insufficient to cause the phenotype of *cpr3::HIS3* mutants. Finally, yeast are normally able to grow on rich medium lacking added carbon source, probably due to amino acid metabolism, which requires mitochondrial function (Fraenkel, 1982). At 37^0C, the

phenotype of the *cpr3::HIS3* mutant MB11-3 on rich medium lacking added carbon source is similar to its phenotype on lactate medium (chapter 3).

It is clear that, if flavocytochrome b_2 is a biochemical target of Cpr3, it cannot be the only target. In sum, the function of Cpr3 is not limited to lactate metabolism, but instead plays a general role in mitochondrial function. This model is consistent with the observed reduced growth of *cpr3::HIS3* mutants on pyruvate and glycerol at 37^{0}C. Although the growth phenotype resulting from *CPR3* inactivation is more severe on lactate than on pyruvate and glycerol, growth on lactate is only slightly better than growth using amino acids as carbon source (unpublished observations). So, cells grown on lactate are probably more sensitive to other environmental stresses, *e. g.*, heat stress, than are cells grown on pyruvate and glycerol.

The inability of *cpr3::HIS3* yeast to grow on lactate is observed at 37^{0}C, but the optimal growth temperature for *S. cerevisiae* is 30^{0}C. The apparent temperature-sensitivity of this observation is consistent with a model that cyclophilins act to catalyze the folding or refolding of proteins *in vivo*. However, wild-type strains are also temperature sensitive during growth on non-fermentable carbon sources, and *cpr3::HIS3* strains exhibit a slower growth phenotype at 30^{0}C (chapters 3 and 4). So, the observed phenotype at 37^{0}C might be the additive result of two deficiencies, one in a Cpr3-dependent pathway, and one in a biological process that is inherently temperature-sensitive on lactate medium. As a result, it is difficult to predict from this temperature sensitivity whether Cpr3 plays a protein folding role in the cell.

A genetic system for identifying cyclophilin-associated factors. I used the yeast system to study cyclophilin function primarily because it is highly amenable to both biochemical and genetic manipulation. This system is most advantageous when a solid phenotype can be identified for the gene of interest. The advantage of using genetic rather than *in vitro* physical methods (e. g., immune co-precipitation, affinity columns) to achieve this goal is that the identification of any candidate targets by these means is obligatorily linked to physiology.

There are some drawbacks to using *in vitro* methodology to identify proteins that interact with the gene product of interest. First, the extraction methods used could produce binding artifacts, as partially- or fully-denatured proteins can aggregate via exposed hydrophobic regions. Second, in the absence of a null cyclophilin phenotype, the mere identification of a binding protein (especially one that is novel) is unlikely to give many clues concerning the function of the gene product of interest. Finally, the affinity of the gene product of interest for a target protein might be too weak to survive the extraction procedure. In fact, if Cpr3 is an enzyme, then one could not expect to isolate Cpr3 with bound substrate.

With a phenotype identified for the *cpr3::HIS3* allele, and after determining that the absence of flavocytochrome b_2 is insufficient to cause the growth defect of *cpr3* mutants, it would have been unwise to then select other specific candidate target proteins to study for their biochemical relationship to Cpr3. This is especially true if one considers that some or all Cpr3 targets could be proteins that have not yet been identified.

I decided to exploit the phenomenon of second-site suppression in order to identify genes that encode biochemical targets of Cpr3. This approach entails identifying genes, other than *CPR3*, that encode proteins that partially or fully restore the ability of *cpr3::HIS3* strains to grow on lactate medium at 37^0C.

The risks associated with using second-site suppression to identify targets of gene products are well-documented (Jarvik and Botstein, 1975). Suppression of the *cpr3::HIS3* phenotype could be obtained by selecting for a gain-of function in a protein not directly linked biochemically with Cpr3. Conceivably, one could identify: 1) a protein that is one or more steps removed in a biochemical pathway from Cpr3; 2) a protein that merely stimulates cell growth, such as a nutrient transporter; or 3) a transcriptional activator for a direct biochemical target of Cpr3.

Finally, the selection for second-site suppressors is most efficient at 37^0C, because at this temperature, suppressors should be easily identified against little or no background. However, it was conceivable to select for a suppressor that overcomes the inherent temperature sensitivity displayed by yeast during incubation on lactate medium. Similar such mutations are known (McCusker, *et al.*, 1994). Despite these caveats, however, I felt that second-site suppression offered the most promise for identifying *in vivo* targets of Cpr3.

I pursued two distinct strategies for achieving second-site suppression of the *cpr3::HIS3* phenotype. The first was to obtain suppression by overexpressing "wild-type" (no known mutations) genes in a *cpr3::HIS3* genetic background. Such genes are known as "high-copy, second-site suppressors". The second approach was to

isolate nuclear genes that caused suppression due to a dominant mutation, one that relieved a protein's dependence on Cpr3 function. Such genes are called "extragenic suppressors" (Jarvik and Botstein, 1975), and the strains harboring such mutations are known as "second-site, pseudo-revertants".

Isolation of *JEN1*, a high-copy suppressor. I identified a novel gene, *JEN1*, that when present on a high-copy plasmid, weakly suppresses the inability of a *cpr3::HIS3* strain to grow on lactate medium at 37^{0}C (chapter 4). The predicted *JEN1* gene product is similar to a family that includes bacterial and yeast carboxylic acid transporters (Culham, *et al.*, 1993; Susatsu, *et al.*, 1985; van Der Rest, *et al.*, 1990; Seol and Shatkin, 1991), the product of a methotrexate resistance gene in *C. albicans* (Fling, *et al.*, 1991), and a mammalian lactate/pyruvate transporter (Garcia, *et al.*, 1994). *JEN1* mRNA is strongly induced by growth on lactate, and very rare or absent in cells grown on glucose or glycerol. Taken together, these results suggest that *JEN1* encodes a lactate transporter.

Predictions of the biochemical relationship of the *JEN1* gene product with Cpr3 are somewhat inconclusive (chapter 4). First, sequence analysis shows that the predicted leader sequence of 21 residues of Jen1 possesses some, but not all, characteristics of a mitochondrial leader sequence. Second, *JEN1* overexpression does not significantly stimulate growth of a *cpr3::HIS3* strain at 30^{0}C. Third, haploid strains carrying a deletion mutation of *JEN1* are impaired for growth on synthetic medium supplemented with L-lactate at 30^{0}C, and on rich medium

supplemented with L-lactate at 37^{0}C. No phenotype was observed for the *jen1::URA3* strains on glucose or glycerol media at either temperature. If the *JEN1* gene product is the major *in vivo* target of Cpr3, then we would expect loss of Jen1 to result in a phenotype similar to that resulting from loss of Cpr3.

There are two possible scenarios that explain these results in terms of the potential biochemical relationship of Jen1 to Cpr3. First, the *JEN1* gene product could be one of several proteins that are direct biochemical targets of Cpr3. In this situation, the observed phenotype of *cpr3::HIS3* mutants could be the cumulative result of deficiencies in several proteins. Therefore, removing only one of these proteins (*e. g.*, the *JEN1* gene product) by mutation would be insufficient to recapitulate the phenotype of *cpr3::HIS3* mutants. Yet, overexpression of one of these proteins (Jen1) can partially relieve the growth deficiency exhibited by the *cpr3::HIS3* mutant strain.

Second, the *JEN1* gene product could be part of a biochemical pathway important for growth on non-fermentable carbon sources, but one not related directly to Cpr3. Yet, its overexpression is still sufficient to rescue the growth phenotype of *cpr3::HIS3* strains. An example of this scenario would be if the predicted *JEN1* gene product was a Cpr3-independent, cell membrane transporter for lactic acid. This is possible because increasing the concentration of lactate in the plates can allow partial growth of *cpr3::HIS3* strains at 37^{0}C (unpublished observations).

The phenotype of the *jen1::URA3* mutation is similar to that of mutations in *CYB2*, a gene encoding a mitochondrial lactate dehydrogenase (chapter 4;

Guiard, *et al.*, 1985). Therefore, the *JEN1* gene product is probably involved primarily in the metabolism of lactate, possibly as a transporter. Since Cpr3 function is not limited to lactate metabolism (chapters 2 and 3), then if the *JEN1* gene product is an *in vivo* target of Cpr3, it is probably one minor target.

Identification of *JEN2-1*, a single-copy suppressor. I identified three *cpr3::HIS3* strains capable of growth on lactate medium at 37^0C (chapter 4). These pseudo-revertants arose spontaneously, at a frequency of *ca.* 2 X 10^{-5}. Segregation analysis indicated that these suppressor mutations are dominant, and occur in nuclear genes. Because laboratory yeast strains are inherently temperature-sensitive (McCusker, *et al.*, 1994), the mutations in these pseudo-revertants could be suppressing the inherent temperature-sensitivity of yeast, rather than the growth-sensitivity specific for loss of Cpr3. So, I applied a phenotypic test to these pseudo-revertant strains to clarify the nature of the suppression: An extragenic suppressor specific for Cpr3 should stimulate growth of *cpr3::HIS3* strains at 30^0C as well as 37^0C, because *cpr3::HIS3* strains exhibit a slower growth phenotype even at 30^0C (chapters 3 and 4).

Two of the pseudo-revertant strains, ED260-1 and ED261-1, exhibited faster growth than the parent strain, MB11-3, at 30^0C. Therefore, the suppressor mutations in these strains are specific for the *cpr3::HIS3* phenotype. Deletion of *JEN1* had no effect on the growth of ED261-2 on D-, L- lactate at 30^0C or 37^0C, indicating that the single-copy suppressor, *JEN2-1*, is not allelic with *JEN1*. The wild-type allele, *JEN2*$^+$, therefore represents a strong candidate for encoding a

protein directly involved in Cpr3 function. It is noteworthy that a dominant mutation in a single gene can ameliorate the *cpr3::HIS3* phenotype, suggesting that there is only one or a few major, direct targets of Cpr3.

Section C: Survey of Recent Findings.

Both genetic (in the case of *ninaA*) as well as biochemical approaches have been exploited by others in the quest to determine cyclophilin function. Unfortunately, little progress in determining the normal physiological roles of cyclophilins has resulted from these efforts.

Only three cases have been reported in which proteins have been found associated with immunophilins in the absence of immunosuppressant drugs. First, a 77 kd protein, termed cyclophilin C-associated protein (CyCAP), was isolated from bone marrow-derived cell line extracts as a protein that binds human cyclophilin C (hCyPC; Friedman and Weissmann, 1991). CsA disrupts this association. The sequence of CyCAP suggests either that it is localized to the secretory pathway, or that it is secreted (Friedman, *et al.*, 1993). CyCAP has a 99 residue, cysteine-rich domain similar to that found in a family of putative, cell-surface-associated receptor proteins exhibiting broad ligand-binding specificities (Dangott, *et al.*, 1989; Kodama, *et al.*, 1990). However, the functions of CyCAP, of the other members of the family, and of the cysteine-rich domain, are unknown. The problem with interpreting these findings is that no physiological process has been associated with either CypC or with CyCAP. Because the physical association of CypC with CyCAP has only been observed *in vitro*, it is possible that this association is an experimental artifact.

Second, two components of a steroid receptor complex are immunophilin homologs (Tai, et. al, 1992; Kieffer, *et al.*, 1992; Ratajczak, *et al.*, 1992). One of

these is a 59 kd FKBP-like protein (FKBP59); the other is a 40 kd cyclophilin-like protein. Both proteins also have calmodulin binding domains, suggesting a role in signal transduction. In the absence of steroid ligand, the receptor forms a complex with CyP-40, FKBP59, and the molecular chaperones Hsp70 and Hsp90. Again, the physiological significance of this association is unknown.

Third, using the yeast two-hybrid system, Luban, *et al.* (1993) demonstrated that human cyclophilins A and B bind to the human immunodeficiency virus type 1 (HIV-1) Gag polyprotein, an association inhibited by CsA. This finding is intriguing, given that the well-known pathology of Acquired Immune Deficiency Syndrome (AIDS) involves loss of T-lymphocytes. The significance of the association of cyclophilins with HIV-gag is unclear. The cyclophilin-gag association does not resemble that of the action of CsA on T-cells, as the CyP-gag complex does not bind calcineurin (Luban, *et al.*, 1993). Perhaps cyclophilins are necessary for the folding of HIV gag, or gag binding precludes the interaction of cyclophilin with an endogenous regulatory ligand.

In none of these cases cited do we see a physical interaction of cyclophilins with another protein, coupled with a physiological consequence. For this reason, progress in determining cyclophilin function has been, at best, quite slow.

Progress in yeast. A total of four cyclophilin genes are known to exist in yeast. *CPR1*, *CPR2*, and *CPR3* all encode proteins with CsA-sensitive PPIase activity (Haendler, *et al.*, 1989; Koser, et. al, 1991; McLaughlin, *et al.*, 1992). It is not yet known if *CPR4* encodes a protein with either PPIase or CsA-binding activities

(Frigerio and Pelham, 1993).

A fifth yeast gene, *SCC3*, has weak homology with other cyclophilin genes (Franco, *et al.* 1991); it is most closely related to the *ninaA* gene of *D. melanogaster* (29% amino acid identity over the 181 residue region common to the two proteins). The predicted proteins of *SCC3* and *ninaA* have a high degree of identity in their amino- and carboxy-terminal domains, but in the domains most highly conserved among the cyclophilin family, the *SCC3* gene product contains many substitutions. The function of the *SCC3* gene product, as well as its CsA binding and PPIase activities, are unknown. Due to its weak homology with the other members of the cyclophilin family, *SCC3* will not be considered further as a cyclophilin in this work.

Little progress has been made in deciphering the functions of the *CPR1*, *CPR2*, and *CPR4* gene products. Cpr1 is a cytosolic protein (Haendler, et. al, 1989), Cpr2 has been reported to be secreted (Zydowsky, *et al.*, 1992), and Cpr4, by virtue of an HDEL carboxy-terminal motif, probably resides in the endoplasmic reticulum (Frigerio and Pelham, 1993). As for *CPR1*, *CPR2*, and *CPR3*, *CPR4* is not essential for viability (Frigerio and Pelham, 1993).

As in T-lymphocytes, Cpr1 mediates some of the pharmacological activity of CsA. *S. cerevisiae* cells treated with CsA are unable to recover from cell-cycle arrest caused by the mating pheromone, α-factor (Foor, *et al.*, 1992). Inactivation of calcineurin in *S. cerevisiae*, while not lethal, also prevents recovery from α-factor arrest (Cyert, *et al.*, 1991; Cyert and Thorner, 1992). *In vitro,* Cpr1 binds calcineurin in the presence of CsA, and the interference by CsA with recovery

from α-factor arrest is overcome by disruption of *CPR1* (Foor, *et al.*, 1992). These results suggest that CsA acts on yeast via an inhibitory complex with Cpr1, consistent with a role of cyclophilin in signal transduction pathways.

Null mutations in *CPR1* and *CPR2* cause a modest decrease in the survival of cells incubated at 48^{0}C (Sykes, *et al.*, 1993). Further, incubation of yeast cells at 37^{0}C causes a 2- to 3-fold stimulation of expression of *CPR1* and *CPR2* mRNA (Sykes, *et al.*, 1992). The *CPR1* 5′ non-coding DNA sequence has a motif resembling a "heat shock element" (a sequence feature of heat shock promoters), and induces reporter gene expression *ca.* 2-fold at 37^{0}C (Sykes, *et al.*, 1993). While yeast are not likely to encounter a temperature as high as 48^{0}C in natural environments, these results imply that one physiological role of Cpr1 and Cpr2 might be to protect cells from temperature extremes (although there is no reason to believe that they do not have functions at normal temperatures). This idea is consistent with a protein folding model for cyclophilins, as protein stability decreases with increasing temperature. However, it will be difficult to use these findings to make progress in discerning the functions of these proteins, for these phenotypes are not absolute. Only 1.1% of wild-type cells survived a 1-hour incubation at 48^{0}C, and heat stress resulted in only a 5-fold decrease in the number of survivors resulting from the mutation in *CPR1* (Sykes, *et al.*, 1993). Further, I have noted an *ca.* 5-fold decrease in *CPR1* mRNA expression upon shift of cells growing on glucose to glycerol medium (unpublished observations). Thus, drawing conclusions from the influence of environmental stresses on cyclophilin gene expression regarding possible functions of cyclophilins is problematic.

Section D: Conclusions.

I have shown in this work that the product of the *CPR3* gene, Cpr3, is a mitochondrial matrix protein that plays a general role in mitochondrial function. Cpr3 is not essential for the signal transduction pathway governing cytochrome gene expression. The physiological role of Cpr3 is independent of temperature, and is only evident during growth on non-fermentable carbon sources. I have identified a novel gene, *JEN1*, that weakly suppresses the phenotype of *cpr3::HIS3* mutants at 37^0C. In addition, I have identified a dominant, nuclear mutation, *JEN2-1*, that overcomes the phenotype of the null *cpr3::HIS3* allele at both 30^0C and 37^0C. *JEN2*$^+$, the wild-type allele of this suppressor, represents a strong candidate for encoding a direct, *in vivo* target of Cpr3.

While not essential for viability, Cpr3 provides yeast cells with a striking selective advantage during growth on non-fermentable carbon sources. Inactivation of *CPR3* has no obvious effect on the growth rate of yeast on glucose (unpublished observations). Cpr3 probably evolved as an activity that optimizes growth under conditions in which glucose is limiting, whereby cells must metabolize either the ethanol from fermentation, or other carbon sources. In competition with wild-type cells in natural environments, a *cpr3* mutant, with a generation time of up to twice as long, would readily become extinct.

A number of questions concerning Cpr3 function remain to be answered. First, what is the identity of the protein encoded by *JEN2*$^+$? What is its biochemical relationship to Cpr3? Despite the promising nature of *JEN2*, the

possibility still exists that it does not encode a protein directly associated with Cpr3. Establishing a direct biochemical link would at a minimum require protein sequence analysis, subcellular localization, mutagenesis, and *in vitro* binding, protein folding, and organelle import experiments. Second, is the *JEN1* gene product a direct target of Cpr3? Answering this question would depend on the same type of experimental verification mentioned above for the product of the *JEN2* gene. Third, are there any additional biochemical targets of Cpr3? Can these putative targets be identified genetically?

The possibility that a transporter molecule, such as the product of the *JEN1* gene, is a biochemical target of Cpr3 is an attractive one, based on a number of independent, experimental observations. Yeast *ade2* mutants are red when grown on rich medium containing glucose. This phenotype is due to accumulation of 5-aminoimidazole ribonucleotide (5-AIR), an intermediate in adenine biosynthesis (Silver and Eaton, 1969; Jones and Fink, 1982). At 37^0, an *ade2* yeast strain carrying the *cpr3::HIS3* allele is pink, while an *ade2 cpr1::URA3 cpr3::HIS3* strain is white (unpublished observations). Enzymes for purine biosynthesis in yeast occur in both the cytosol and mitochondria (Jones and Fink, 1982). Perhaps cyclophilins are involved in transport of a precursor of 5-AIR between the cytosol and mitochondria.

FK-506 prevents growth of yeast auxotrophic for tryptophan or histidine, possibly by inhibiting the transport of these amino acids (Heitman, *et al.*, 1993). Two high-copy suppressors of this effect are similar to a family of amino acid transporters (Heitman, *et al.*, 1993). These findings suggests that amino acid

transporter function is dependent on an FK-506 binding protein. Yeast has two known FKBPs (Wiederrecht, *et al.*, 1991; Nielsen, *et al.*, 1992), but neither of these isoforms is involved in FK-506-sensitive amino acid metabolism (Heitman, *et al.*, 1993).

CsA inhibits the Ca^{2+}-induced swelling of rat liver and heart mitochondria (Davidson and Halestrap, 1990). This effect is thought to be mediated by inhibition of an adenine nucleotide transporter (Halestrap and Davidson, 1990). Finally, in *Xenopus leavis* oocytes, CsA inhibits the surface expression of injected genes encoding ligand-gated ion channels (Helakar, *et al.*, 1994). This effect is suppressed by overexpression of an injected rat cyclophilin gene.

One final issue concerning the normal physiological functions of cyclophilins is the *in vivo* relevance of PPIase activity. As mentioned above, loss-of function mutations in the *D. melanogaster ninaA* gene have been indirectly associated with loss of proline isomerase activity. Cpr3 is reported to possess proline isomerase activity (McLaughlin, *et al.*, 1992). So, similar types of experiments could be carried out directly on Cpr3, using the experimental system that I have developed.

The *S. cerevisiae CPR3* gene is only one of a large family of cyclophilin genes, which might be necessary for a large, diverse set of biological processes. The chief attraction of the system that I have developed in this work is that it is predicated on a strong observable phenotype, in an organism with an extremely well-characterized genetic system. I have developed a very tractable experimental system for studying cyclophilin function.

References

Bierer, B. E., Somers, P. K., Wandless, T. J., Burakoff, S. J., & Schreiber, S. L. (1990a). Probing immunosuppressant action with a nonnatural immunophilin ligand. *Science* **250**, 556-559.

Bierer, B. E., Mattila, P. S., Standaert, R. F., Herzenberg, L. A., Burakoff, S. J., Crabtree, G. R., & Schreiber, S. L. (1990b). Two distinct signal transduction pathways in T-lymphocytes are inhibited by complexes formed between an immunophilin and either FK-506 or rapamycin. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 9231-9235.

Clipstone, N. A. and Crabtree, G. R. (1992). Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* **357**, 695-697.

Culham, D. E., Lasby, B., Marangoni, A. G., Milner, J. L., Steer, B. A., van Nues, R. W., & Wood, J. M. (1993). Isolation and sequencing of *Escherichia coli* gene *proP* reveals unusual structural features of the osmoregulatory proline/betaine transporter, ProP. *J. Mol. Biol.* **229**, 268-276.

Cyert, M. S., Kunisawa, R., Kaim, D., & Thorner, J. (1991). Yeast has homologs (*CNA1* and *CNA2* gene products) of mammalian calcineurin, a calmodulin-regulated phosphoprotein phosphatase. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 7376-

7380.

Cyert, M. S. & Thorner, J. (1992). Regulatory subunit (*CNB1* gene product) of yeast Ca^{2+}/calmodulin-dependent phosphoprotein phosphatases is required for adaptation to pheromone. *Mol. Cell Biol.* **12**, 3460-3469.

Danielson, P. E., Forss-Petter, S., Brow, M. A., Calavetta, L., Douglass, J., Milner, R. J., & Sutcliffe, J. G. (1988). p1B15: A cDNA clone of the rat mRNA encoding cyclophilin. *DNA* **7**, 261-267.

Dangott, L. J., Jordan, J. E., Bellet, R. A., & Garbers, D. L. (1989). Cloning of the mRNA for the protein that cross-links to the egg peptide speract. *Proc. Natl. Acad. Sci. U. S. A.* **86**, 2128-2132.

Davidson, A. M. & Halestrap, A. P. (1990). Partial inhibition by cyclosporin A of the swelling of liver mitochondria *in vivo* and *in vitro* induced by sub-micromolar $[Ca]^{2+}$, but not by butyrate. *Biochem. J.* **268**, 147-152.

Fischer, G., Wittman-Liebold, B., Lang, K., Kiefhaber, T., & Schmid, F. X. (1989). cyclophilin and peptidyl-prolyl *cis-trans* isomerase are probably identical proteins. *Nature* **337**, 476-478.

Flanagan, W. M., Corthesy, B., Bram, R. J., & Crabtree, G. R. (1991). Nuclear

association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature* **352**, 803-807.

Fling, M. E., Kopf, J., Tamarkin, A., Gorman, J. A., Smith, H. A., & Koltin, Y. (1991). Analysis of a *Candida albicans* gene that encodes a novel mechanism for resistance to benomyl and methotrexate. *Mol. Gen. Genet.* **227**, 318-329.

Foor, F., Parent, S. A., Morin, N., Dahl, A. M., Ramadan, N., Chrebet, G., Bostian, K. A., & Nielsen, J. B. (1992). Calcineurin mediates inhibition by FK-506 and cyclosporin of recovery from α-factor arrest in yeast. *Nature* **360**, 682-684.

Forsburg, S. L. & Guarente, L. (1989). Communication between mitochondria and the nucleus in regulation of cytochrome genes in the yeast *Saccharomyces cerevisiae*. *Ann. Rev. Cell Biol.* **5**, 153-180.

Fraenkel, D. G. (1982). Carbohydrate metabolism. In The molecular and cellular biology of the yeast Saccharomyces: Metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Franco, L., Jiménez, A., Demolder, J., Molemans, F., Fiers, W., & Contreras, R. (1991). The nucleotide sequence of a third cyclophilin homologous gene from *Saccharomyces cerevisiae*. *Yeast* **7**, 971-979.

Friedman, J. & Weissman, I. (1991). Two cytoplasmic candidates for immunophilin action are revealed by affinity for a new cyclophilin: One in the presence and one in the absence of CsA. *Cell* **66**, 799-806.

Friedman, J., Trahey, M., & Weissman, I. (1993). Cloning and characterization of cyclophilin C-associated protein: A candidate natural cellular ligand for cyclophilin C. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 6815-6819.

Frigerio, G. & Pelham, H. R. B. (1993). A *Saccharomyces cerevisiae* cyclophilin resident in the endoplasmic reticulum. *J. Mol. Biol.* **233**, 183-188.

Garcia, C. K., Goldstein, J. L., Pathak, R. K., Anderson, R. G. W., & Brown, M. S. (1994). Molecular characterization of a membrane transporter for lactate, pyruvate, and other monocarboxylates: Implications for the Cori cycle. *Cell* **76**, 865-873.

Guiard, B. (1985). Structure, expression and regulation of a nuclear gene encoding a mitochondrial protein: The yeast L(+)-lactate cytochrome c oxidoreductase (cytochrome b_2). *EMBO J.* **4**, 3265-3272.

Halestrap, A. P. & Davidson, A. M. (1990). Inhibition of Ca^{2+}-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl *cis-trans*

isomerase and preventing it interacting with the adenine nucleotide translocase. *Biochem. J.* **268**, 153-160.

Haendler, B., Keller, R., Hiestand, P. C., Kocher, H. P., Wegmann, G., & Movva, N. R. (1989). Yeast cyclophilin: Isolation and characterization of the protein, cDNA, and gene. *Gene* **83**, 39-46.

Harding, M. W., Galat, A., Uehling, D. E., & Schreiber, S. L. (1989). A receptor for the immunosuppressant FK506 is a *cis-trans* peptidyl-prolyl isomerase. *Nature* **341**, 758-760.

Heitman, J., Koller, A., Kunz, J., Henriquez, R., Schmidt, A., Movva, N. R., & Hall, M. N. (1993). The immunosuppressant FK506 inhibits amino acid import in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13**, 5010-5019.

Helakar, S. A., Char, D., Neff, S., & Patrick, J. (1994). Prolyl isomerase requirement for the expression of functional homo-oligomeric ligand-gated ion channels. *Neuron* **12**, 179-189.

Jarvik, J. & Botstein, D. (1975). Conditional-lethal mutations that suppress genetic defects in morphogenesis by altering structural proteins. *Proc. Natl. Acad. Sci. U. S. A.* **72**, 2738-2742.

Jones, E. W. & Fink, G. R. (1982). Regulation of amino acid and nucleotide biosynthesis in yeast. In The molecular and cellular biology of the yeast Saccharomyces: Metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Kieffer, L. J., Seng, T. W., Li, W., Osterman, D. G., Handschumacher, R. E., & Bayney, R. M. (1992). Cyclophilin-40, a protein with homology to the p59 component of the steroid receptor complex. *J. Biol. Chem.* **268**, 12303-12310.

Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P., & Krieger, M. (1990). Type I macrophage scavenger receptor contains α-helical and collagen-like coiled coils. *Nature* **343**, 531-535.

Koser, P. L., Sylvester, D., Livi, G. P., & Bergsma, D. J. (1990). A second cyclophilin-related gene in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **18**, 1643.

Koser, P. L., Bergsma, D. J., Cafferkey, R., Eng, W-K., McLaughlin, M. M., Ferrara, A., Silverman, C., Kasyan, K., Bossard, M. J., Johnson, R. K., Porter, T. G., Levy, M. A., & Livi, G. P. (1991). The *CYP2* gene of *Saccharomyces cerevisiae* encodes a cyclosporin A-sensitive peptidyl-prolyl *cis-trans* isomerase with an N-terminal signal sequence. *Gene* **108**, 73-80.

Liu, J. & Walsh, C. T. (1990). Peptidyl-prolyl *cis-trans* isomerase from *Escherichia*

coli: A periplasmic homolog of cyclophilin that is not inhibited by cyclosporin A. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 4028-4032.

Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I., & Schreiber, S. L. (1991a). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**, 807-815.

Lodi, T. & Guiard, B. (1991). Complex transcriptional regulation of the *Saccharomyces cerevisiae CYB2* gene encoding cytochrome b_2: *CYP1* (*HAP1*) activator binds to the *CYB2* upstream activation site UAS1-B2. *Mol. Cell. Biol.* **11**, 3762-3772.

Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V., & Goff, S. P. (1993). Human immunodeficiency virus type 1 gag protein binds to cyclophilins A and B. *Cell* **73**, 1067-1078.

McCaffrey, P. G., Luo, C., Kerppola, T. K., Jain. J., Badalian, T. M., Ho, A. M., Burgeon, E., Lane, W. S., Lambert, J. N., Curran, T., Verdine, G. L., Rao, A., & Hogan, P. G. (1993). Isolation of the Cyclosporin-sensitive T cell transcription factor NFATp. *Science* **262**, 750-754.

McCusker, J. H., Clemons, K. V., Stevens, D. A., & Davis, R. W. (1994). Genetic characterization of pathogenic *Saccharomyces cerevisiae* isolates. *Genetics* **136**,

1261-1269.

McLaughlin, M. M., Bossard, M. J., Koser, P. L., Cafferkey, R., Morris, R. A., Miles, L. M., Strickler, J., Bergsma, D. J., Levy, M. A., & Livi, G. P. (1992). The yeast cyclophilin multigene family: Purification, cloning, and characterization of a new isoform. *Gene* **111**, 85-92.

Nielsen, J. B., Foor, F., Siekierka, J. J., Hsu, M.-J., Ramadan, N., Morin, N., Shafiee, A., Dahl, A. M., Brizuela, L., Chrebet, G., Bostian, K. A., & Parent, S. A. (1992). Yeast FKBP-13 is a membrane-associated FK506-binding protein encoded by the nonessential gene *FKB2*. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 7471-7475.

O' Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J., & O'Neill, E. A. (1992). FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. *Nature* **357**, 692-694.

Ondek, B., Handy, R. W., Baker, E. K., Stamnes, M. A., Shieh, B.-H., & Zuker, C. S. (1992). Genetic dissection of cyclophilin function. Saturation mutagenesis of the *Drosophila* cyclophilin homolog *ninaA*. *J. Biol. Chem.* **267**, 16460-16466.

Ratajczak, T., Carrello, A., Mark, P. J., Warner, B. J., Simpson, R. J., Moritz, R. L., & House, A. K. (1992). The cyclophilin component of the unactivated

estrogen receptor contains a tetratricopeptide repeat domain and shares identity with p59 (FKBP59). *J. Biol. Chem.* **268**, 13187-13192.

Schreiber, S. L. (1992). Immunophilin-sensitive protein phosphatase action in cell signaling pathways. *Cell* **70**, 365-368.

Seol, W. & Shatkin, A. J. (1991). The *Escherichia coli kgtP* gene encodes an α-ketoglutarate transporter. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 3802-3806.

Silver, J. M. & Eaton, N. R. (1969). Functional blocks of the ad_1 and ad_2 mutants of *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **34**, 301-305.

Susatsu, M., Misra, T. K., Chu, L., Laddaga, R., & Silver, S. (1985). Cloning and DNA sequence of a plasmid-determined citrate utilization system in *Escherichia coli*. *J. Bacteriol.* **164**, 983-993.

Sykes, K., Gething, M.-J., & Sambrook, J. (1993). Proline isomerases function during heat shock. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 5853-5857.

Tai, P.-K. K., Albers, M. W., Chang, H., Faber, L. E., & Schreiber, S. L. (1992). Association of a 59-kilodalton immunophilin with the glucocorticoid receptor complex. *Science* **256**, 1315-1318.

Tropschug, M., Barthelmess, I. B., & Neupert, W. (1989). Sensitivity to cyclosporin A is mediated by cyclophilin in *Neurospora crassa* and *Saccharomyces cerevisiae*. *Nature* **342**, 953-955.

van der Rest, M. E., Schwarz, E., Oesterhelt, O., & Konings, W. N. (1990). DNA sequence of a citrate carrier of *Klebsiella pneumoniae*. *Eur. J. Biochem.* **189**, 401-407.

Wiederrecht, G., Brizuela, L., Elliston, K., Sigal., N. H., & Siekierka, J. J. (1991). *FKB1* encodes a nonessential FK506-binding protein in *Saccharomyces cerevisiae* and contains regions suggesting homology to the cyclophilins. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 1029-1033.

Zydowsky, L. P., Ho, S. I., Baker, C. H., McIntyre, K., & Walsh, C. T. (1992). Overexpression, purification, and characterization of yeast cyclophilins A and B. *Protein Sci.* **1**, 961-969.

Appendix A. Publication of Chapter 1

The material in chapter 1 was published exactly as written in *Proc. Natl. Acad. Sci. U. S. A.* 89, 11169-11173 (1992)). The title and author affiliations are as follows:

A Yeast Cyclophilin Gene Essential For Lactate Metabolism At High Temperature

Classification: Genetics

(Key terms: mitochondria/temperature sensitive)

Edward S. Davis[*†], Andrea Becker[‡¶], Joseph Heitman[§||], Michael N. Hall[§], and Miles B. Brennan[*††]

[*]Unit on Genomics, Clinical Neurogenetics Branch, National Institute of Mental Health, Building 10, Room 4N320, Bethesda, MD 20892. Telephone: (301)402-1769 FAX:(301)402-2140

[†]Department of Zoology, University of Maryland, College Park, MD 20742

[‡]Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 N. Torrey Pines Rd., La Jolla, CA 92037

[§]Department of Biochemistry, Biocenter of the University of Basel, CH-4056 Basel, Switzerland

[¶]Present address: Department of Cell Biology, Howard Hughes Medical Institute, Yale University, 333 Cedar St., New Haven, CT 06510

[||]Present address: The Rockefeller University, 1230 York Ave., New York, NY 10021

Appendix A (continued)

††To whom reprint requests should be addressed.

Abbreviations: CsA, cyclosporin A; PPIase, peptidyl prolyl cis-trans isomerase.

EMBL Accession Number X56962

Acknowledgements

We thank Dr. Ute Hochgeschwender for encouragement and advice in initiating this project, Dr. J. Gregor Sutcliffe for his support of the early stages of this work, Dr. Marco Colombini for helpful discussions, and Drs. Werner Klee and Howard Nash for critical review of the manuscript.

Appendix B. Publication of Chapter 3

The material in chapter 3 has been submitted to *The Journal of Biological Chemistry*, with the following title and author affiliations:

The Yeast Cyclophilin Cpr3 is a Mitochondrial Matrix Protein Important for Mitochondrial Function.

Edward S. Davis[*†] and Miles B. Brennan[*]

[*]Unit on Genomics, Clinical Neurogenetics Branch, National Institute of Mental Health, Building 10, Room 4N320, Bethesda, MD 20892 USA

[†]Department of Zoology, University of Maryland, College Park, MD 20742 USA

Acknowledgements

We thank Dr. Werner Klee for critical reading of the manuscript, and Dr. Brian Martin for use of the Betagen counter.

Appendix C. Publication of Chapter 4

The material in chapter 4 has been submitted to *Molecular and Cellular Biology*, with the following title and author affiliations:

Genetic Analysis of Cyclophilin Function: Identification of Loci That Suppress a Null Phenotype of the Yeast *CPR3* Gene.

Edward S. Davis[*†] and Miles B. Brennan[*]

[*]Unit on Genomics, Clinical Neurogenetics Branch, National Institute of Mental Health, Building 10, Room 4N320, Bethesda, MD 20892 USA

[†]Department of Zoology, University of Maryland, College Park, MD 20742 USA

Acknowledgements

We thank Dr. Brian Martin for oligonucleotide synthesis. We also thank Dr. Michael Lichten for instruction and use of equipment for tetrad dissection and micromanipulation.