Title
Adaptation to the loss of signal recognition particle dependent protein targeting in yeast

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Adaptation to the Loss of Signal Recognition Particle Dependent Protein Targeting in Yeast

by

Sarah C. Mutka

DISSERTATION

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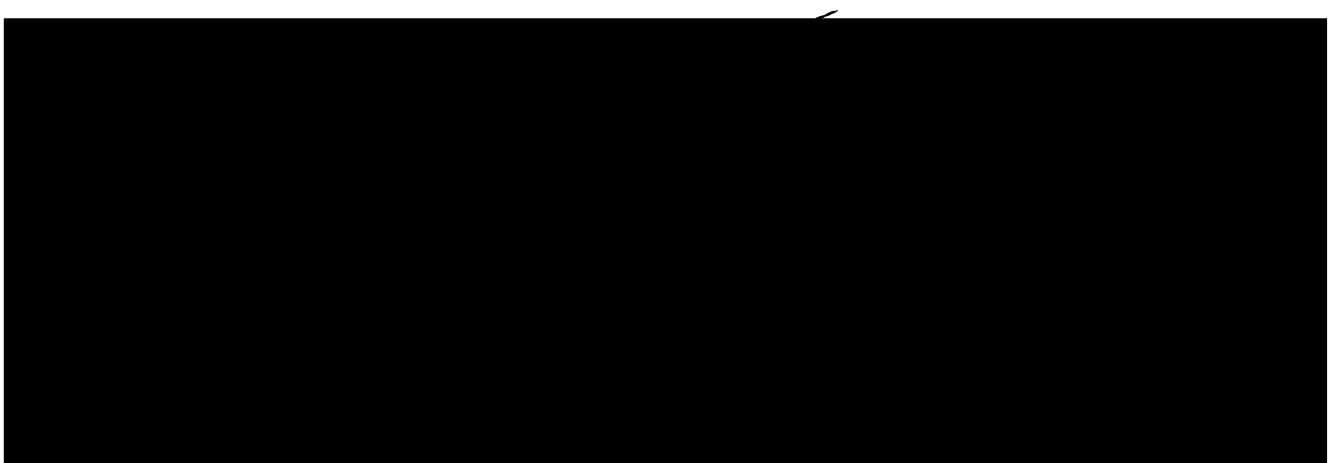

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This thesis is dedicated to my parents, John and Virginia Mutka

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Abstract

In this thesis I examine the response of the yeast *Saccharomyces cerevisiae* to the loss of the signal recognition particle (SRP)-dependent protein targeting pathway. I have used two inducible mutants that block the SRP pathway to demonstrate that cells mount a physiological response to the loss of the SRP pathway that includes specific changes in global gene expression. These expression changes describe the intricate intertwining of various cell responses to a potentially devastating event. I also examined three genomic deletions in different components of the SRP pathway to describe gene expression changes specific to the different factors in the pathway. In agreement with previous studies, I found that upon inducing the loss of the SRP pathway, SRP-dependent protein translocation is initially blocked, and cell growth is considerably slowed. Concomitantly, gene expression changes include the induction of heat shock genes and the repression of protein synthesis genes. Remarkably, within hours, the efficiency of protein sorting improves while cell growth remains slow in agreement with the persistent repression of protein synthesis genes. My results suggest that heat shock gene induction serves to protect cells from mislocalized precursor proteins in the cytosol, whereas reduced protein synthesis helps to regain efficiency in protein sorting by reducing the load on the protein translocation apparatus. Taken together, these results provide another example of a link between the secretory pathway, protein synthesis, and stress responses and suggest that cells trade speed in cell growth for fidelity in protein sorting to adjust to life without SRP.

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CHAPTER I:

Introduction

INTRODUCTION

Eukaryotic cells are highly compartmentalized, containing multiple membrane-bound organelles designed to segregate specialized functions of the cell into isolated regions. Many different membranes are capable of supporting protein translocation and a variety of different mechanisms and pathways exist for these purposes. As the entry point for the secretory pathway, proteins destined for the Golgi complex, and most proteins of the vacuole, the endoplasmic reticulum (ER) is a major site for protein translocation across membranes within the cell (Rapoport et al., 1996). However, the mitochondrion (Herrmann and Neupert, 2000), the chloroplast (Chen and Schnell, 1999), the peroxisome (Erdmann et al., 1997), and the vacuole (Klionsky and Ohsumi, 1999) all are capable of supporting translocation across their membranes.

Although many distinct mechanisms exist to accommodate these various protein translocation reactions, four common principles are evident (reviewed in Wickner et al., 1991). These include signal sequences directing the translocated proteins, receptor proteins in the membranes, an energy requirement for the translocation reaction, and cytosolic chaperones to allow translocated proteins to maintain a translocation-competent state. During my numerous years as a graduate student, the focus of my work has been on these common principles of how proteins are delivered to their proper location within the cell. Initially, this work focused on protein sorting to the intermembrane space of the mitochondrion, and eventually the direction of my work turned to how cells survive in the absence of the signal recognition particle (SRP)-dependent protein targeting pathway. This thesis will describe this work and the conclusions that can be drawn from it.

Protein Import into the Mitochondrion

For some organelles, such as mitochondria or chloroplasts, separate protein import pathways exist to deliver the appropriate proteins directly to these locations. In the case of mitochondria, an organelle bounded by two membranes, the central steps of protein import and the common principles shared with other translocation reactions are clear. The vast majority of mitochondrial proteins are synthesized on cytosolic ribosomes, and these proteins contain targeting signal sequences at their amino terminus. A subset of cytosolic HSP70 proteins interact with these proteins to keep them in an unfolded state compatible with import (reviewed in Craig et al., 1993). These proteins, through interactions with their signal sequences, can then bind receptor subunits of the TOM (translocase of the outer membrane) complex on the outer membrane of the mitochondrion (Gratzer et al., 1995; Hachiya et al., 1995; Kiebler et al., 1993). Upon binding, proteins to be imported entirely into the inner most compartment of the mitochondrion are translocated across the protein subunits which comprise the general insertion pore of the TOM complex (Sollner et al., 1992) (reviewed in Lithgow, 2000) and across a TIM (translocase of the inner membrane) complex in the inner membrane (reviewed in Herrmann and Neupert, 2000). These translocation reactions require both ATP and a membrane potential (Glick et al., 1992; Schneider et al., 1994). On arrival at their final destination, the signal sequences are cleaved by proteases within the mitochondrion (Pollock et al., 1988; Yang et al., 1988).

The signal sequences that direct proteins to mitochondria have been closely scrutinized for unique characteristics. Generally, there is no sequence similarity for targeting signals to any given compartment. Therefore, targeting information must be and is contained

in features such as charge distribution, hydrophobicity, and/or other structural information (Ng et al., 1996; von Heijne and Abrahmsen, 1989; Wickner et al., 1991). In the case of mitochondrial presequences, these signals are generally 10-70 residues in length and rich in positively charged and hydroxylated residues. The sequences generally can form amphiphilic α-helices exposing basic residues on one face and hydrophobic residues on the other face. After the protein is properly directed to its destination, these presequences are cleaved by metalloproteases residing in the matrix compartment (von Heijne et al., 1989).

Protein targeting to the Endoplasmic Reticulum

With a few exceptions, for secreted proteins, plasma membrane proteins, lumenal and membrane proteins of the ER, the Golgi complex, and the vacuole the first step towards their ultimate destinations begins with targeting to the ER membrane. In every organism examined, a signal recognition particle (SRP)-dependent, co-translational pathway has been identified which is responsible for the targeting of many of these proteins to the membrane (reviewed in Walter and Johnson, 1994). In addition to the SRP-dependent pathway, alternative SRP-independent post-translational pathways have also been identified in bacteria and in yeast for targeting of other proteins (reviewed in Brodsky, 1998). Furthermore, homologs to proteins involved in these pathways have been identified in higher eukaryotes (see for example Meyer et al., 2000). In both SRP-dependent and SRP-independent targeting pathways, the common requirements (signal sequence, receptors, energy, and chaperones) of translocation pathways discussed above hold true, and I will summarized those requirements and differences between the two targeting pathways here.

As mentioned previously, signal sequences generally have very little conservation in their primary sequences. However, signal sequences directing proteins to the ER share several structural characteristics. These include a positively charged amino terminus of 1 to 5 amino acids, a 7 to 15 residue core of hydrophobic amino acids, and a 3 to 7 residue C-terminal region containing a cleavage site for a signal peptidase (reviewed in Brodsky, 1998). Furthermore, an examination of signal sequences in yeast has allowed us to conclude that the degree of hydrophobicity of the core is responsible for determination of the translocation pathway to be used. Signal sequences of SRP-dependent substrates display significantly greater hydrophobicity than signal sequences of SRP-independent substrates (Ng et al., 1996).

For both translocation pathways, the requirement for chaperones in the cytosol also holds true. In SRP-independent targeting, proteins are fully translated before they are targeted to the ER membrane. In such cases, cytosolic chaperones associate with the proteins to maintain them in an unfolded, translocation competent state. Proteins shown to be involved in this process include the SSA family of cytosolic Hsp70s and Ydj1 (Caplan et al., 1992; Chirico et al., 1988; Deshaies et al., 1988). In the case of SRP-dependent targeting, SRP obviates the need for traditional cytosolic chaperones. When signal sequence-containing nascent proteins emerge from the translating ribosomes, they are recognized by SRP. This causes a pause in translation until the SRP-ribosome-nascent chain complex is properly targeted to the membrane. In this sense, SRP could be considered a chaperone of this targeting reaction.

Both translocation pathways have distinct protein complexes that function as receptors at the ER membrane. In the case of SRP-dependent targeting, the SRP receptor

(SR) consists of two proteins, SRα and SRβ, anchored to the ER membrane. Interaction between SR and SRP causes the SRP-ribosome-nascent chain complex to be directed to the ER membrane. In SRP-independent targeting, the Sec62/Sec63 complex functions as a receptor. This complex contains Sec62, Sec63, Sec71, and Sec72, and it is generally thought to be associated with the trimeric Sec61 translocon complex universal to both types of translocation, although it may exist as a separate complex as well (reviewed in Rapoport et al., 1996).

The SRP-SR interaction introduces a requirement for energy in the SRP-dependent pathway in the form of GTP hydrolysis. Upon SRP-ribosome-nascent chain complex binding to SR, GTP hydrolysis allows dissociation of SRP from the SR and recycling of the SRP for further rounds of targeting. Energy is required in the cytosol for cytosolic Hsp70 proteins as well as in the ER lumen for the SRP-independent pathway. There, a lumenal Hsp70 protein, Kar2 is involved in driving protein translocation in an ATP-dependent manner (reviewed in Brodsky, 1998).

The yeast *Saccharomyces cerevisiae* provides an excellent model system to study both of these targeting and translocation pathways as well as their interactions. Our hope was that we might be able to understand the cross-talk between the pathways by examining cells in which one pathway is compromised. In order to do this, we focused on the SRP-dependent targeting pathway and how cells survive in its absence, and I will provide more specific background on the SRP pathway in yeast here.

SRP-dependent protein targeting in yeast

SRP was initially purified and shown to be involved in translocation in mammalian cells. The particle was determined to contain one RNA molecule and six protein subunits named after their molecular weights (SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72)(Walter and Blobel, 1980). The SRP receptor was also identified and shown to consist of two proteins, SRα (72 kDa) and SRβ (30 kDa) (Tajima et al., 1986). Later, a gene was identified in *Saccharomyces cerevisiae* which encodes a protein homologous to SRP54 of mammalian cells (Hann et al., 1989). Using antibodies to the yeast SRP54, this protein was shown to be part of a ribonucleoprotein complex with a major cytoplasmic RNA, scR1. Although these components are not essential for cell growth, they were shown to be required for efficient protein translocation across the ER membrane (Hann and Walter, 1991).

Other protein components of the SRP in yeast were soon identified. Sequence analysis of a mutation in the *SEC65* gene demonstrated that this gene product is related to the SRP19 subunit, and experimentation showed that it is associated with SRP54 (Hann et al., 1992). Later, purification of yeast SRP by immuno-affinity chromatography identified the remaining four yeast SRP subunits. Disruption of any of these genes resulted in indistinguishable phenotypes from those described for SRP54 (Brown et al., 1994). Genes encoding the SRP receptor subunits SRα (Ogg et al., 1992) and SRβ (Ogg et al., 1998) were also cloned by homology to the mammalian proteins and shown to play similar roles in the targeting pathway.

Although deletion of any of the components of the SRP pathway in yeast is not lethal, cell growth is severely affected causing growth three to six fold slower than in wild type cells (Hann and Walter, 1991; Ogg et al., 1992). Surprisingly, although the particle has been

shown to play an essential role in protein translocation, deletions in these proteins do not display dramatic translocation defects of SRP-dependent substrates. Rather, extended time courses in protein depletion experiments show that cells adapt to the loss of the SRP-pathway in terms of accumulation of untranslocated proteins (Ogg et al., 1992), but the molecular basis of this adaptation was unknown.

In this thesis, I describe my efforts to characterize the molecular basis of adaptation to the loss of the SRP-dependent protein targeting pathway in yeast. In Chapter 2, I describe biochemical, genetic, and genomic experiments that allow us to draw general conclusions about the major changes in all adapted cells. In Chapter 3, I extend the analysis of genomic experiments to describe transcriptional differences in mutations in different components of the SRP pathway. In an appendix, I describe a first project aimed at isolating mutants which fail to properly sort proteins to the intermembrane space of the mitochondrion.

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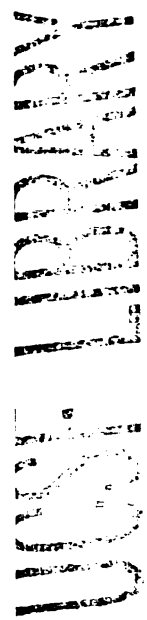
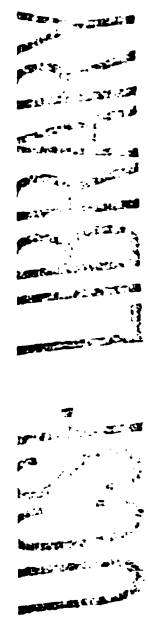
CHAPTER II:

A Multifaceted Physiological Response Allows Yeast to Adapt to the Loss of the SRP-dependent Protein Targeting Pathway



ABSTRACT

Translational control has recently been recognized as an important facet of adaptive responses to various stress conditions. We describe the adaptation response of the yeast *Saccharomyces cerevisiae* to the loss of one of two mechanisms to target proteins to the secretory pathway. Using inducible mutants that block the signal recognition particle (SRP) pathway, we find that cells demonstrate a physiological response to the loss of the SRP pathway that includes specific changes in global gene expression. Upon inducing the loss of the SRP pathway, SRP-dependent protein translocation is initially blocked, and cell growth is considerably slowed. Concomitantly, gene expression changes include the induction of heat shock genes and the repression of protein synthesis genes. Remarkably, within hours, the efficiency of protein sorting improves while cell growth remains slow in agreement with the persistent repression of protein synthesis genes. Our results suggest that heat shock gene induction serves to protect cells from mislocalized precursor proteins in the cytosol, whereas reduced protein synthesis helps to regain efficiency in protein sorting by reducing the load on the protein translocation apparatus. Thus, we suggest that cells trade speed in cell growth for fidelity in protein sorting to adjust to life without SRP.



INTRODUCTION

All proteins destined for the secretory pathway must first be targeted to the endoplasmic reticulum (ER). In mammalian cells, this targeting reaction primarily occurs co-translationally via the signal recognition particle (SRP) pathway. Both the components and the mechanism of SRP-dependent protein targeting are conserved in every organism studied to date from bacteria to eukaryotic cells. Without translocation, proteins would quickly accumulate in the cytosol, and the hydrophobic nature of many translocated membrane proteins would cause massive protein aggregation and severe stress for the cell.

SRP and the SRP-dependent protein targeting pathway have been well characterized (for reviews see (Brodsky, 1998) and (Walter and Johnson, 1994)). In the yeast *Saccharomyces cerevisiae*, SRP consists of six protein subunits and a small RNA (Brown et al., 1994; Hann and Walter, 1991). Briefly, SRP-dependent targeting begins as nascent chains emerge from the ribosome, and those with ER-specific signal sequences are recognized and bound by SRP. The SRP-ribosome-nascent chain complex is then directed to the ER membrane through an interaction between SRP and the SRP receptor (SR), which consists of two proteins, SRα and SRβ, anchored to the ER membrane. The ribosome-nascent chain complex is released from SRP-SR and directed to the Sec61 membrane translocon, allowing co-translational translocation of the protein across the ER membrane to proceed (Johnson, 1999).

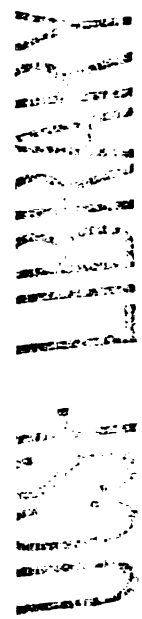
In addition to the SRP pathway, many organisms have evolved alternative, SRP-independent protein targeting pathways. In yeast, the core proteins of this pathway are Sec62,

Sec63, Sec71, and Sec72. These proteins associate with the Sec61 translocon, forming a membrane complex required for this alternative translocation pathway (Deshaies et al., 1991; Deshaies and Schekman, 1989; Rothblatt et al., 1989). For SRP-independent targeting, chaperones are required in order to keep cytosolic precursor proteins in an unfolded, translocation-competent state. Proteins implicated for this role include the SSA chaperone family and Ydj1 (Caplan et al., 1992; Chirico et al., 1988; Deshaies et al., 1988). Directed by information contained in their hydrophobic signal sequences, targeting of some proteins, such as dipeptidyl aminopeptidase B (DPAP-B) or Kar2, is strongly SRP-dependent, whereas the targeting of others, such as carboxypeptidase Y (CPY), is SRP-independent (Brown et al., 1994; Ng et al., 1996).

The SRP pathway is essential in all organisms examined to date except the yeast *S. cerevisiae* (Hann and Walter, 1991). Deletion of any component of the SRP targeting pathway displays indistinguishable phenotypes, indicating that each of these individual deletion mutations results in the disruption of the entire pathway. Yeast strains lacking the SRP pathway are exceedingly sick; they grow into heterogeneously sized colonies, growing three to six fold slower than isogenic wildtype strains (Hann and Walter, 1991; Ogg et al., 1992). Moreover, transcriptional shut-off of SRP pathway components results in an accumulation of untranslocated SRP-dependent proteins (Brown et al., 1994; Ogg et al., 1992). Thus, although SRP is not essential in *S. cerevisiae*, the loss of the SRP pathway has severe negative consequences for the cell.

Surprisingly, although depletion of SRP proteins causes an accumulation of many untranslocated precursor proteins, strains with genomic deletions of SRP genes do not display dramatic translocation defects of SRP-dependent proteins. Indeed, extended time

courses with inducible depletion of SRP components demonstrated that cells "adapt" to the absence of the SRP-dependent pathway as monitored by the reduction of untranslocated precursor proteins (Ogg et al., 1992). Here we address the molecular basis of adaptation to begin to understand the adaptive response mounted by *S. cerevisiae* to survive the loss of SRP-mediated protein translocation.



MATERIALS AND METHODS

Strains Used in this Study

W303 (*MATα, leu2-3-112, his3-11, trp1-1, ura3-1, can1-100, ade2-1*); SMY246 (W303 [rho-]); SMY211 (W303, pDN66) (plasmid from Davis Ng, Penn State University); SMY212 (W303, pGal*SRP54*); SMY226 (W303, *hsf::LEU2,* pDN66, pHF35 (*HSF1*^{C}) (knockout construct from (Sorger and Pelham, 1988)); YTH119 (W303, *MATα, srp102::URA3*) from T. Hu, University of California, San Francisco; SMY286 (YTH119, pTH123), SMY288 (YTH119, pSO462); SMY268 (W303 *MATα, SEC63-prA HIS3, URA3*) (sec63prA tag (Beckmann et al., 1997)); SMY266 (W303 *MATa, SEC63-prA, srp102::URA3,* pSO462); SMY284a (W303 *MATa, srp54::neo*) from Gustavo Pesce, University of California, San Francisco; SOY60 (W303, *MATα, scr1::HIS3*)(Ogg et al., 1992).

Plasmids used in this study

pGalSRP54 (Gal-*SRP54*, URA3, CEN4/ARS1) (Hann and Walter, 1991); pDN66 (Gal-*SRP54*^{dn}, URA3, CEN4/ARS1) from Davis Ng, see below; pHF35 (HF/1-40Δ147 (*HSF1*^{C}), TRP1, CEN6/ARSH4) (Sorger, 1990); pTH123 (*SRP102*-3xFlag, TRP1, CEN6/ARSH4) from Dr. T. Hu, University of California, San Francisco; pSO462 (*srp102*(K51I)-HA, TRP1, CEN6/ARSH4) (Ogg et al., 1998); pSM110 (pGal*SRP54*^{dn}, TRP1, CEN6/ARSH4); pSM131 (Gal-*SRP54*, TRP1, CEN6/ARSH4)

Construction of pDN66

The SRP54 G201A mutation was generated using the Kunkel method (Kunkel et al., 1987). The full length *SRP54* gene was inserted into the vector pRS313 (Sikorski and Heiter, 1989) at *Xba* I and *Bam*HI to generate the phagemid pDN2. pDN2 single stranded DNA was purified from phage produced from transformed CJ236 cells following infection with the helper phage VCSM13. Second strand synthesis was performed using a mutagenic primer changing glycine 201 to alanine (5' -GATACTTCAGCAAGGCATCA-3'). The resulting DNA was transformed into DH5α cells and the mutant plasmid (pDN50) was isolated from transformants and confirmed by DNA sequence analysis. pDN66 was constructed by subcloning a *BstE*II/*Sal* I fragment from pDN50 to replace a similar fragment in pGALSRP54 (Hann and Walter, 1991).

Isotopic Labeling and Nonnative Immunoprecipitation

Metabolic labeling and immunoprecipitation assays were performed as described (Ng and Walter, 1996) except that cells were labeled for 7 minutes. All yeast cultures were grown and labeled at 30°C except for *srp102*(K51I) cells, which were grown and labeled at 23°C or 37°C as indicated. Monospecific polyclonal antisera were used for immunoprecipitation of endogenous protein. Anti-DPAP-B antiserum was generously provided by Tom Stevens (University of Oregon, Eugene, OR). Quantitation was performed with a Molecular Dynamics (Sunnyvale, CA) Storm 840 imager and ImageQuant Software. Untranslocated

precursor is represented as a ratio of precursor versus total protein recovered which controls for expression changes of the substrate or loading differences.

Purification of the Sec63 Complex

The Sec63 complex was purified as described (Ogg et al., 1998) with the following modifications. Cells were grown to 0.3-0.8 OD_{600}U/ml in medium lacking methionine followed by labeling at a density of 3 OD_{600}U/ml with 30 μCi/ OD_{600}U of [^{35}S] Pro-mix cell labeling mix (Amersham) for 45 min to 1 h with aeration. Labeled cells were treated exactly as described except for the composition of the lysis buffer (LB; 50 mM Hepes_KOH pH 7.5, 200 mM sorbitol, 100 mM KOAc pH 7.5, 5 mM $Mg(OAc_2)$, 5 mM DTT, 1 mM PMSF, 1μg/ml pepstatin and leupeptin), and membranes were solubilized in digitonin (GHBD; 10% glycerol, 3% digitonin, 50 mM Hepes_KOH pH 7.5, 200 mM sorbitol, 400 mM KOAc pH 7.5, 5 mM $Mg(OAc_2)$, 5 mM DTT, 1mM PMSF, 1 μg/ml pepstatin and leupeptin). The detergent extracts were used immediately without freezing. Sec63prA protein complexes were purified in GHBD with 40 μl Sepharose CL-4B and 0.5 μl-1 μl IgG Sepharose 6 Fast Flow/ OD_{600}U cells (Amersham Pharmacia Biotech, Uppsala, Sweden) for 3 h at 4°C with rotation. After extensive washing with GHBD, proteins were eluted from the IgG Sepharose with 100 mM glycine pH 2.0, TCA precipitated, and analyzed by SDS-PAGE.

Genomic Arrays: Sample Preparation and Hybridization

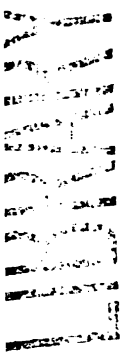
Strains were grown to mid-log phase in YPD or synthetic media as indicated. At the indicated time points, cells were centrifuged at room temperature and snap frozen in liquid nitrogen. Total RNA was prepared by the SDS-hot phenol/bead lysis method (Kingston, 1997), and mRNA was isolated using the polyATtract system (Promega) according to the manufacturer's instructions. Amino-allyl dUTP (aadUTP) was incorporated during reverse transcription of 2-3 μg poly(A)$^+$ RNA, primed with $pd(T)_{12-18}$ (Amersham) and pdN_6 (Gibco BRL) as described (DeRisi et al., 1997) except the nucleotide final concentrations were: 500 μM for dATP, dCTP, and dGTP, 300 μM dTTP, 200 μM aadUTP (Sigma #A0410). After reverse transcription, reactions were adjusted to 0.2 M NaOH, 0.1 M EDTA and incubated for 15 min at 65°C for hydrolysis of RNA, followed by neutralization with Tris.HCl pH 7.4 to 0.33 M. Tris was removed from the reaction by washing with Centricon-30 microconcentrators (Amicon) as described (DeRisi et al., 1997). Monofunctional NHS-ester Cy3 or Cy5 (Amersham) was coupled to the cDNA via the incorporated aadUTP in 0.1 M sodium bicarbonate buffer pH 9.0 in the dark at room temperature for 1h. The reactions were quenched by adjusting to 1.33 M hydroxylamine and incubating for 15 min at room temperature in the dark. Cy3 and Cy5 reactions were combined, and unincorporated dye was removed with the Qia-quick PCR purification kit (Qiagen) according to the manufacturer's instructions. cDNAs were hybridized to prepared microarrays as described (DeRisi et al., 1997) (see also http://www.microarrays.org/protocols.html).

Genomic Data Analysis and Categorization

Microarrays were visualized using a GenePix scanner (Axon), and fold changes in mRNA levels relative to control samples were determined using GenePix analysis software. ORFs of interest were placed into categories based on functional category descriptions in the Yeast Protein Database (YPD) (http://www.proteome.com (Costanzo et al., 2000)).

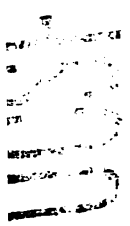
Quantitation of the Rate of [^{35}S]-methionine Incorporation

Quantitation of the rate of [^{35}S]-methionine incorporation into protein was performed as described (Ogg and Walter, 1995) except that at each time point cells were plunged into ice cold azide buffer (AB; 20 mM NaN_3, 50 mM NaCl) and snap frozen in liquid nitrogen. The cells were then quick thawed, harvested, and washed once in AB before lysis in TCA.



Online Supplemental Material

Datasets of the genomic expression experiments are available online at the Walter lab web pages (http://walterlab.ucsf.edu). The data are expression ratios formatted as text files which can be opened in various programs including Microsoft Excel. The datasets include: 1) "all srp ratio.txt": the complete genomic dataset with expression ratios of all SRP experiments and 2) "704 ORFs.txt": the subset of the complete dataset which is described and categorized in Table 1.



RESULTS

Cells adapt to the loss of the SRP Pathway by a reversible, physiological process

To address the molecular basis of the adaptive response to the loss of SRP, we developed two independent means to disable the SRP pathway quickly and reversibly. The first approach depends on a plasmid-borne, galactose-inducible dominant negative allele of *SRP54* (*SRP54^{dn}*), one of the subunits of the signal recognition particle. The dominant negative allele in *SRP54^{dn}* is a mutation in the second G-box domain (G201A) (Bernstein et al., 1989) that, by analogy to other GTPases, is predicted to interfere with GTP hydrolysis. Although the mechanism of action for Srp54^{dn} remains to be characterized, the dominant negative effect is likely to arise from a block of GTP hydrolysis, resulting in Srp54^{dn} locked onto the SRP receptor, sequestering the SRP receptor into an inactive pool (Rapiejko and Gilmore, 1992). After induction of *SRP54^{dn}*, cells displayed phenotypes identical to SRP deletion strains including their characteristic slow growth and variable colony size (data not shown)

We tested the effects of *SRP54^{dn}* induction on protein translocation as a function of time with a pulse-labeling and immunoprecipitation experiment. Cells were grown in selective media containing raffinose, and then switched to galactose-containing media to induce *SRP54^{dn}* or, as a control, *SRP54*. In galactose, the *SRP54^{dn}* cells grow four-fold slower than the control strain (data not shown). At 0, 4, 8, 12, and 16 h after induction, cells were pulse-labeled, and an SRP-dependent protein substrate, Kar2, was immunoprecipitated and analyzed by SDS-PAGE. Translocation defects are monitored by following the lack of

protein processing modifications normally made upon entry into the ER. For Kar2, translocation defects were inferred from the accumulation of a more slowly migrating precursor form, indicating the signal sequence has not been cleaved (Fig. 1A, pre-Kar2). The precursor form of Kar2 reflects a defect in translocation rather in processing as demonstrated previously (Ogg et al., 1992). To demonstrate that adaptation is not limited to a single substrate, we tested another SRP-dependent substrate, DPAP-B. For this protein, translocation defects are inferred from the appearance of a faster migrating unglycosylated precursor form (Fig. 1B and C, pre-DPAP-B). The translocation defect peaked at about 4 h after *SRP54^{dn}* induction with the accumulation of approximately 60% untranslocated Kar2 (Fig.1A, lane 7), diminished at later time points, and persisted at about 25% untranslocated Kar2 (Fig. 1A, lanes 8 – 10). As expected, cells expressing wildtype *SRP54* showed no growth or protein translocation defects indicating that the observed defect was not simply due to an overproduction of Srp54.

DPAP-B showed a similar profile (Fig. 1B). Four hours after induction of *SRP54^{dn}*, as much as 90% DPAP-B was detected as untranslocated pre-DPAP-B (Fig.1B, lane 7). Again, the amount of accumulated precursor protein diminished to nearly wildtype levels within 8 to 12 hours. The cells' response to SRP loss was therefore biphasic: an immediate accumulation of untranslocated SRP-dependent precursor proteins (peaking around 4 h after SRP loss) followed by a reduction of untranslocated precursor proteins due to adaptation.

To assess the generality of adaptation, we used a second method to disrupt the SRP pathway. We took advantage of a strain in which the chromosomal copy of *SRP102* (SRβ) has been disrupted but contains a plasmid with a temperature-sensitive allele, *srp102(K51I)* (Ogg et al., 1998). At 37°C, these cells grow approximately six-fold slower than wildtype

cells (Ogg et al., 1998). As shown in Fig. 1C, a shift to the non-permissive temperature led to the accumulation of pre-DPAP-B after the 2 and 4 h time points, while at later time points precursor protein rapidly returned to levels close to those observed in wildtype cells (Fig. 1C, lanes 9 - 10), reminiscent of the biphasic response observed after induction of the dominant negative allele of *SRP54* (Fig. 1A and B).

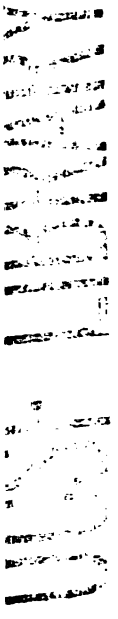
Previous results indicated that adaptation is a physiological response and not due to a suppressor mutation. This argument was based on genetic evidence that, once backcrossed and sporulated, SRP or SRP receptor deletion strains which are constitutively adapted showed no evidence of inheritance of the adapted state (Ogg et al., 1992). To address this issue more directly, we took advantage of the inducible *SRP54^{dn}* mutant to monitor adaptation over multiple rounds of switching the SRP pathway on and off. We induced expression of Srp54^{dn} and monitored the effects on translocation of Kar2 as described above. As expected, we observed the transient accumulation of pre-Kar2 followed by adaptation (Fig. 2, lanes 2 and 3). After blocking the SRP pathway for 16 h, the cells were switched back to growth under non-inducing conditions for 24 h. After this time, we again induced Srp54^{dn} expression and observed the initial accumulation of pre-Kar2, followed by adaptation over a four to eight hour period (Fig. 2, lanes 7 – 10). Thus, the recovered cells behaved indistinguishably from wildtype cells that were never deprived of a functional SRP pathway. This result confirms that genetic suppression does not play a role in adaptation to the loss of the SRP pathway.

The composition of the translocon remains unchanged after adaptation

We next sought to identify physiological changes occurring in response to adaptation that are important for allowing cells to cope with the loss of the SRP pathway. In *S. cerevisiae*, the SRP-independent post-translational translocation pathway has been well characterized (for review see (Rapoport et al., 1996). Both translocation pathways are thought to use the same translocon composed of Sec61 and its associated subunits Sss1 and Sbh1, but different accessory proteins are required. For SRP-dependent translocation, these proteins include the heterodimeric SRP receptor, and for post-translational translocation, these proteins include a complex of Sec63, Sec62, Sec71, and Sec72 (Green et al., 1992; Ng et al., 1996; Panzner et al., 1995; Rothblatt et al., 1989). Because most protein substrates studied show some degree of promiscuity in their choice of protein translocation pathways (Ng et al., 1996), we considered the possibility that in adapted cells, SRP-dependent proteins might be translocated post-translationally with enhanced efficiency due to a structural change in the translocon itself. To explore this notion, we determined whether the composition of the translocon is changed in any quantitative or qualitative way in response to the loss of the SRP pathway.

To this end, we purified translocon complexes to examine their protein composition and abundance in wildtype and adapted cells. We used a strain containing a protein A-tagged version of Sec63 to allow for a one-step affinity isolation (Aitchison et al., 1995; Beckmann et al., 1997). We disrupted the SRP pathway either by expressing the *SRP54^{dn}* allele or by a temperature shift of cells bearing the *srp102(K51I)* mutation, and allowed cells to adapt. Translocon complexes were purified by extracting microsomes with digitonin, a mild detergent that has been shown to preserve the integrity of the translocon (Panzner et al., 1995), and isolating the translocon complexes via protein A-tag binding to IgG Sepharose.

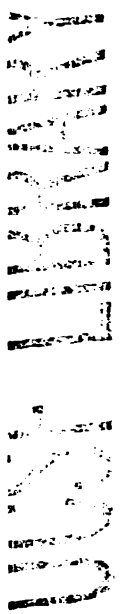
As expected, in the wildtype controls, Sec61, Sec62, Sec71, and Sec72 co-purify with the Sec63 fusion protein and are the major proteins observed (Fig. 3, lanes 2 and 4) With this gel system, we did not detect Sss1 or Sbh1 because of their smaller size. In the adapted cells, we see an indistinguishable pattern of proteins (Fig. 3, compare lanes 2-3 and 4-5). Consistent with the similarities at the protein level, no up-regulation of mRNAs encoding these proteins was observed in adapted cells according to genomic expression array data (see supplemental genomics data). From these results, we conclude that neither the abundance nor the composition of the translocon is adjusted as cells adapt to the loss of the SRP pathway. These results suggest that if SRP-dependent proteins are translocated via the post-translational pathway in adapted cells, they do so using translocon complexes present under normal growth conditions.



Transcriptional responses to the loss of SRP

Expression levels of a limited number of chaperone proteins have been shown to be induced in a strain depleted of Srp54 (Arnold and Wittrup, 1994). To expand on this observation more comprehensively, we determined the global changes in the transcriptional program of the cell that accompany SRP loss and adaptation. For this purpose, we used DNA microarrays to screen the expression changes of all yeast open reading frames (ORFs) under these conditions. The DNA microarrays were generated by PCR amplification of 6,352 yeast ORFs and printing on a glass microscope slide (DeRisi et al., 1997). At various time points following disruption of the SRP pathway, mRNA was extracted from the cells, converted into cDNA, and fluorescently labeled. Reference samples were labeled with Cy3 (green),

and experimental samples were labeled with Cy5 (red). For each time point, the experimental probes were mixed with the appropriate reference probes, and the mixture was hybridized to a microarray. The relative abundance of each mRNA was then measured by comparison of the relative intensity of the red and green signals, giving a measure of the relative expression of each ORF at various times during the process of SRP-depletion and adaptation. We represent relative expression levels visually with color blocks (Figs. 4A and 6A). Shades of green represent levels of repression and shades of red represent induction relative to the reference strain.



Using this approach, we analyzed the consequences of blocking the SRP pathway by either induction of *SRP54*^{dn} or temperature shift of SRβ ^{ts}. To minimize variance due to the differences in growth conditions necessary to induce the SRP pathway mutations, we subjected reference strains to the same conditions in order to subtract out many of these differences. For example, the galactose inducible *SRP54*^{dn} cells were compared to galactose inducible *SRP54*^{wt} cells to control for both the carbon source shift and protein overexpression. Similarly, the SRβ^{ts} cells were compared to SRβ^{wt} cells also grown at 37°C to control for the temperature shift. In both cases, we monitored transcriptional changes as a function of time, and comparison of the data from the two experimental systems allowed us to focus on major changes common to SRP loss. Thus observed changes would be more likely to represent physiological responses to SRP loss rather than to reflect changes inherent in the changes of growth conditions. In addition to the time courses following SRP loss in the two inducible systems, we analyzed the long-term consequences of genomic deletions of three different components of the SRP pathway, Srp54, SRP RNA (encoded by *SCR1*), and SRβ.

For this study, we limited our analyses to ORFs that experienced at least 2-fold induction or repression in both time courses. We examined each time point and selected ORFs for which at least 3 time points from both experimental systems met the cutoff criteria. From the 6,352 ORFs examined, 704 ORFs (11% of the total genome) met these criteria with two thirds being repressed and one third being induced (see supplemental genomics data). The ORFs were grouped according to cellular function, and these groups are summarized in Table 1.

Although a very broad spectrum of genes are either repressed or induced in response to the loss of the SRP pathway, changes in three major transcriptional programs stood out: 1) a large number of genes encoding chaperones and heat shock factors were induced (30 genes), 2) many genes encoding ribosomal proteins were repressed (76 genes), and 3) mitochondrial and/or energy generation genes are repressed (35 genes, for discussion of this category see Table 1).

Chaperone/heat shock induction

Induction of a limited number of heat shock proteins was previously observed upon SRP loss (Arnold and Wittrup, 1994). Our results showed that this transcriptional program was induced and included a large number of genes encoding chaperones and other heat shock proteins. Plotting expression levels of these genes versus time showed that the sharp, peak induction of these genes coincided with the peak of untranslocated proteins accumulated in the cytosol (2 h for the SRβ^{ts} cells, 4 h for the *SRP54*^{dn} cells, Fig. 4B and C). The steady state levels of these mRNAs then decreased over time. In the case of SRP pathway depletion

using a temperature shift of SRβ^{ts} cells, the expression levels at late time points became comparable to those in a control strain also grown at 37°C, representing a sustained heat shock response (Fig.4B, 12h). Sustained upregulation, however, was also observed at the late time points after *SRP54*^{dn} induction where experimental and control cells were maintained at 30°C (Fig. 4C, 16h and 31h). Representing the fully adapted state, the induction of chaperone/heat shock genes was also observed in several genomic deletions of SRP and SR components (Fig. 4A, gene deletions in the three right most columns).

To address how the heat shock/chaperone inductive response corresponds in scope and magnitude to a genuine heat shock response, we compared our data to a published data set for a 39°C heat shock treatment (Roth et al., 1998), in which 263 genes were judged to have been induced relative to the control. We found that, upon the disruption of the SRP pathway, 10% of these genes were induced, at peak expression levels, at least 3-fold greater than in the heat shock experiment, 50% of these genes showed induction of similar magnitude, and 40% were not induced to heat shock levels. Thus, the chaperone and heat shock gene induction observed in response to the loss of the SRP pathway substantially overlaps with a heat shock response, yet it is not identical.

We next asked if the observed induction of heat shock proteins would be sufficient for adaptation to the loss of SRP. If sufficient, adaptation should be facilitated in cells in which heat shock proteins are constitutively expressed at elevated levels. To test this hypothesis, we used a constitutively active form of Hsf1 (*HSF1*^{C}), the transcription factor controlling genes with promoters containing a heat shock element (HSE). When expressed, Hsf1^{C} is sufficient to cause a persistently high level of heat shock protein expression (greater than two-fold higher than expression due to heat shock), without a need for elevated

temperature or any other inducing stress (Sorger, 1990; Sorger and Pelham, 1988). We disrupted the SRP pathway in strains expressing $Hsf1^{C}$ by induction of $SRP54^{dn}$ and compared the amount of untranslocated protein to a wildtype strain after 4 hours of $SRP54^{dn}$ expression (Fig. 5). Even with its constitutively elevated level of heat shock proteins, however, the $HSF1^{C}$ strain showed translocation defects indistinguishable from those observed in wildtype strains. Given this result, we conclude that the elevated level of heat shock induction observed in this strain is not sufficient to result in or accelerate adaptation.

We next wanted to determine whether elevated expression levels of heat shock proteins are necessary for adaptation. Because *HSF1* is an essential gene, we used strains expressing a mutant version of *HSF1* (HF/40Δ147-583) that is not inducible (Sorger, 1990). Analysis of HF/40Δ147-583 cells showed a marginal, if any, deficiency in the ability to adapt to the loss of the SRP pathway when compared to an isogenic wildtype strain (data not shown). Similar experiments with knockouts or conditional alleles of individual chaperones (*SSA1, SSA2, YDJ1*^{ts}, *HSP104, HSP82,* and *HSP26/42* double knockout) showed either no effect or only very marginal effects on adaptation. Taken together these results suggest that adaptation either relies on redundant signaling pathways or heat shock proteins that have not been tested or that the elevated levels of heat shock proteins are not required for adaptation.

Repression of Ribosome Biogenesis

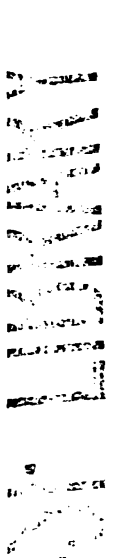
The second and most comprehensive transcriptional program in response to SRP loss is the repression of genes responsible for protein synthesis. Seventy-one different ribosomal proteins, for example, are downregulated at least 2-fold in response to the loss of SRβ. In addition, a variety of other genes encoding components of the protein synthesis machinery

are repressed, including genes encoding elongation and initiation factors and rRNA and tRNA processing proteins. The same effect was observed upon induction of *SRP54^{dn}*, when over 100 genes encoding ribosomal proteins were downregulated at least two-fold during the time course (Fig. 6A and B). In contrast to the biphasic up-regulation of chaperone/heat shock genes described above, ribosomal protein genes show a monotonic repression profile following loss of SRP pathway function (Fig. 6B).

As expected, a significant reduction in the rate of total protein synthesis resulted as a consequence of the transcriptional repression of protein synthesis genes. After induction of *SRP54^{dn}*, we pulse-labeled cells with [^{35}S]-methionine and measured incorporation of the radioactive amino acid over time into total protein. [^{35}S]-methionine uptake was comparable between the wildtype and dominant negative strains (data not shown). We compared the incorporation rates after 4 hours of disruption of the SRP pathway (unadapted cells) and after16 hours (adapted cells). We observed a 9-fold decrease in the rate of protein production after 16 h of *SRP54^{dn}* expression compared to after 4h, whereas the wildtype controls exhibited a 3-fold decrease presumably due to shift to galactose (data not shown). Thus as predicted by the genomic expression data, protein synthesis is repressed in adapted cells.

We next asked whether decreasing protein synthesis could suppress the translocation defects observed early after SRP pathway loss before cells become adapted. To this end, we treated *SRP54^{dn}* cells with a range of sublethal cycloheximide concentrations (Ogg and Walter, 1995) to artificially cause a reduction in protein synthesis and monitored translocation defects 4 h time point after induction of *SRP54^{dn}*. At the maximal cycloheximide concentration used (2 μM), [^{35}S]-methionine incorporation was reduced19-fold as compared to untreated cells (data not shown). As shown in Figure 7, we observed a

significant dosage-dependent decrease in the relative amount of untranslocated proteins, suggesting that reduced protein synthesis can contribute to the cell's ability to adapt to the loss of SRP.



DISCUSSION

In these studies, we have characterized the adaptive response to the loss of the SRP pathway in *S. cerevisiae*. Using inducible mutants, we have demonstrated that adaptation is a reversible, physiological response that occurs rapidly upon loss of SRP or SR function. Null mutants of SRP pathway components display the same adapted phenotype, demonstrating that adaptation is not due to the use of partial SRP function in our inducible systems. Although translocating proteins in adapted cells are likely to make extensive use of alternative targeting pathways, apparently no change in the wildtype state of the translocon is required to do so. However, we observed a highly complex set of transcriptional changes, including the induction of heat shock genes and the repression of genes involved in protein synthesis.

Concurrent with the accumulation of untranslocated proteins in the cytosol, we observed a large induction in heat shock gene expression including chaperones implicated in protein translocation (such as the SSA genes encoding members of the HSP70 chaperone family). In addition to this inductive response, we also observed ribosomal repression upon disruption of the SRP pathway. Whereas the expression of ribosomal genes was consistently low throughout adaptation, heat shock gene induction peaked early during the response, concomitant with the amount of untranslocated proteins in the cell. Heat shock gene expression then persisted at lower levels throughout adaptation. Chaperones and heat shock proteins are likely to be required to maintain proteins in an unfolded state until they are either translocated in an SRP-independent manner or degraded in the cytosol. Thus, it is plausible that the spike in chaperone expression is necessary to accommodate an initially high load of

untranslocated protein. Perhaps reduced protein synthesis diminishes the need for these proteins during the adaptation phase. In this view, heat shock proteins play a protective role rather than being instrumental for the process of adaptation *per se*: transient induction of chaperones may aid in clearing the cytosol of untranslocated proteins, whereas a persistent decrease in the cell's protein synthesis capacity may stop the problem at its source. Late in adaptation, cells may reach a translation rate at which they can accommodate all proteins that must enter the ER in the absence of SRP. This would explain why, over time, SRP$^-$ strains regain the ability to target SRP-dependent proteins to the ER, but still grow more slowly than wildtype strains.

Connections between regulation of ribosome biogenesis and the secretory pathway have been observed before. In *Escherichia coli*, for example, suppressor analysis of conditional *sec* mutations yielded primarily mutations that compromise protein synthesis, and it has been proposed that the decreased synthesis of precursor proteins relieves the lethal burden placed on the mutant Sec machinery (Lee and Beckwith, 1986; Danese et al., 1995; Oliver, 1985). In yeast, reduction in protein synthesis by cycloheximide treatment suppresses the temperature-sensitive effects of the SRP mutant, *sec65-1* (Ogg and Walter, 1995). Furthermore, it was demonstrated that defects in the secretory pathway at any point from the ER membrane translocon to the trans-Golgi network cause a significant repression of ribosomal proteins and RNAs (Mizuta and Warner, 1994; Nierras and Warner, 1999). Translational regulation has also been suggested to play a role in cell survival during the unfolded protein response (UPR) by reducing the protein load on the folding machinery during stress (Harding et al., 2000). It seems likely that repression of ribosome biogenesis is

having a similar effect of supporting adaptation by reducing the protein load on alternative translocation pathways.

The exact mechanism of targeting and translocation of proteins in the absence of SRP remains unclear. Thus, we do not know if it is the reduction in ribosomal capacity (as indicated by the genomic expression data), a reduced elongation rate, or a both that allows for survival in the absence of the SRP pathway. Here, we have only shown that a reducction in translational elongation alone can partially alleviate translocation defects caused by the loss of SRP in non-adapted cells. Many proteins studied show some degree of flexibility in their choice of protein translocation pathway (Ng et al., 1996), and it seems plausible that protein translocation is accommodated post-translationally in the absence of SRP. However, if the observed decrease in protein synthesis includes slowing elongation, it remains possible that some proteins may be translocated co-translationally even in the absence of the SRP targeting pathway. Parallels may exist in the mechanism of protein import into mitochondria where it has been argued that the relative kinetics of translation and import may allow a subset of protein import to occur co-translationally (Lithgow, 2000).

Other models invoking SRP-independent co-translational translocation are also conceivable. Recent studies suggest that a substantial fraction of large ribosomal subunits remain membrane bound after termination of protein synthesis (Potter and Nicchitta, 2000) and that translation of signal sequence-bearing proteins initiating on such membrane-bound ribosomal subunits can directly access the translocon in the absence of SRP receptor function (Seiser and Nicchitta, 2000). We have shown that the abundance of translocons does not change in response to the loss of the SRP pathway, yet based on the genomic expression data ribosomal capacity is reduced. Thus, the ratio of ribosomes to translocons in SRP-depleted

cells is proportionally lower than in wildtype cells. It is thus conceivable that more translational initiation events occur on membrane-bound ribosomes, increasing the chance of proper targeting in the absence of a functional SRP pathway.

In this study, we focused on trends of only two transcriptional programs revealed by the genomic expression data. In light of the vast number of known genes and uncharacterized ORFs that are induced or repressed in the absence of the SRP pathway, it seems unlikely that the combined effects of chaperone up-regulation and decreased protein synthesis capacity describe the full extent of the adaptive process. Rather, a multiplicity of physiological changes may contribute to survival in the absence of the SRP pathway, including other responses revealed by the genomic expression data as well as processes regulated at the translational or post-translational level. More sophisticated genetic tools will need to be employed to provide focus on the key causal changes that allow cells to survive such a severe stress.

It is unclear what role, if any, protein degradation plays in the adaptive response. It is possible that what appears to be improved translocation efficiency in adapted cells is, in whole or part, due to increased specific degradation of accumulated precursor proteins. Pulse-chase experiments aiming to determine the half-lives of untranslocated proteins during adaptation have yielded divergent results depending on the SRP disruption system chosen. After temperature shift of $SR\beta^{ts}$ cells, for example, pre-Kar2 had similar half-lives throughout the adaptation time course ($T_{1/2}$ = 89 min at 2 h; 94 min at 12 after temperature shift; S. M. and P. W., unpublished observations), suggesting that increased degradation of precursor proteins is not responsible for the apparent improvement of translocation efficiency observed in these cells. In contrast, upon induction of $SRP54^{dn}$, we observed an increased

rate of pre-Kar2 disappearance ($T_{1/2}$ = 40 min at 4 h; 23 min at 16 after temperature shift; S. M. and P. W., unpublished observations) that was not accompanied by a corresponding increase in translocated protein, i.e., could not be accounted for by post-translational protein translocation. This suggests that pre-Kar2 is degraded at an increased rate in the adapted cells. Thus, there may be different ways in which cells can cope with SRP loss that may depend on growth conditions or other factors.

With the sole exception of *S. cerevisiae*, the SRP pathway is essential in all organisms examined to date. Even in *S. cerevisiae* however, it is clear from growth and protein translocation phenotypes, as well as from the vast number of gene expression changes characterized here, that the loss of the SRP pathway causes enormous stresses for the cell. Our data suggest that, in the absence of SRP, protein synthesis is repressed which may be instrumental for allowing cell survival but at the same time giving rise to a much reduced growth rate. The cell may therefore trade speed for fidelity, as a compromise when the SRP pathway is no longer functional. Indeed, this recourse may be a very general principle that cells use for surviving a variety of stresses that, for cells growing in the wild, are likely to be transient. Translational regulation is now emerging as an important mechanism for surviving stresses, such as defects in the secretory pathway (Mizuta and Warner, 1994; Nierras and Warner, 1999) or accumulation of unfolded proteins (Harding et al., 2000) and, as argued here, may contribute to adaptation to the loss of the SRP pathway.

ACKNOWLEDGMENTS

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Table 1: Summary of 704 ORFs Responsive to the Loss of SRP

CATEGORY	# of ORFs	# INDUCED	#REPRESSED	% of total
Chaperone/heat shock	31	30	1	4.4
Protein synthesis	77	1	76	11
Mitochondrial/energy generation†	36	1	35	5
Metabolism*	97	29	68	14
Transcription	18	7	11	2.5
RNA processing	6	5	1	0.8
DNA replication, recombination, repair, structure	18	3	15	2.5
Protein modification	11	1	10	1.6
Protein degradation	7	5	2	1
Vesicular transport	11	4	7	1.6
Signaling	7	1	6	1
Cell wall/structural	12	2	10	1.7
Mating/budding	4	0	4	0.6
Cell cycle	4	2	2	0.6
Other	5	1	4	0.7
Uncharacterized ORFs	360	148	212	51
TOTAL	704	240	464	100 %

*23 small molecule transporters, 74 involved in the metabolism of amino acids (20), carbohydrates (17), nucleotides (15), lipids/fatty acids (17), phosphate (2), and others (3).

† Cells disrupted for SRP function rapidly lose the ability to grow on non-fermentable carbon sources, i.e., unless selective pressure is applied to the contrary, they become rho$^-$. The reason for this tendency is unknown; it is not a prerequisite for survival as cells can be forced to retain mitochondrial function if they are continuously grown on non-fermentable carbon sources. We previously characterized protein translocation defects of rho- strains following SRP-depletion and found that these strains can also adapt (Ogg et al., 1992). We therefore conclude that a loss of respiratory function is not responsible for adaptation.

FIGURE LEGENDS

Figure 1. Protein translocation returns to wildtype efficiency in the absence of functional SRP54 or SRβ over time. (A) Kar2 immunoprecipitation in the SRP54^{dn} system. Translocation of Kar2 was compared in strains containing either a pGAL-*SRP54^{wt}* plasmid (SMY212) or a pGAL-*SRP54^{dn}* plasmid (SMY211). Cells were grown to mid-log phase in raffinose-containing selective synthetic medium lacking uracil, then shifted to the comparable galactose-containing media. Cells were labeled with [^{35}S]-methionine for 7 min, and harvested at the indicated times. Lysates at each time point were immunoprecipitated with anti-Kar2 and analyzed by SDS-PAGE followed by autoradiography. Lumenal (Kar2) and cytosolic precursor (pre-Kar2) forms are indicated. The amount of precursor protein relative to lumenal protein at each time point was quantified and graphed. The graph and error bars for the *SRP54^{dn}* strain reflect the average and standard deviation of nine experiments. (B) DPAP-B immunoprecipitation in the SRP54^{dn} system. Experiments were carried out and analyzed as described for panel A. Lumenal (DPAP-B) and cytosolic precursor (pre-DPAP-B) forms are indicated. (C) DPAP-B immunoprecipitation in the SRβ^{ts} system: Translocation of DPAP-B in a wildtype strain (SMY286, *srp102::URA3*, pTH123) or a strain containing the *srp102(K51I)* ts allele of *SRP102* (SMY288, *srp102::URA3* , pSO462). Cells were grown to mid-log phase in YPD at 23°C then shifted to 37°C to induce the SRβ^{ts} allele. Cells were labeled and immunoprecipitated as described in (A).

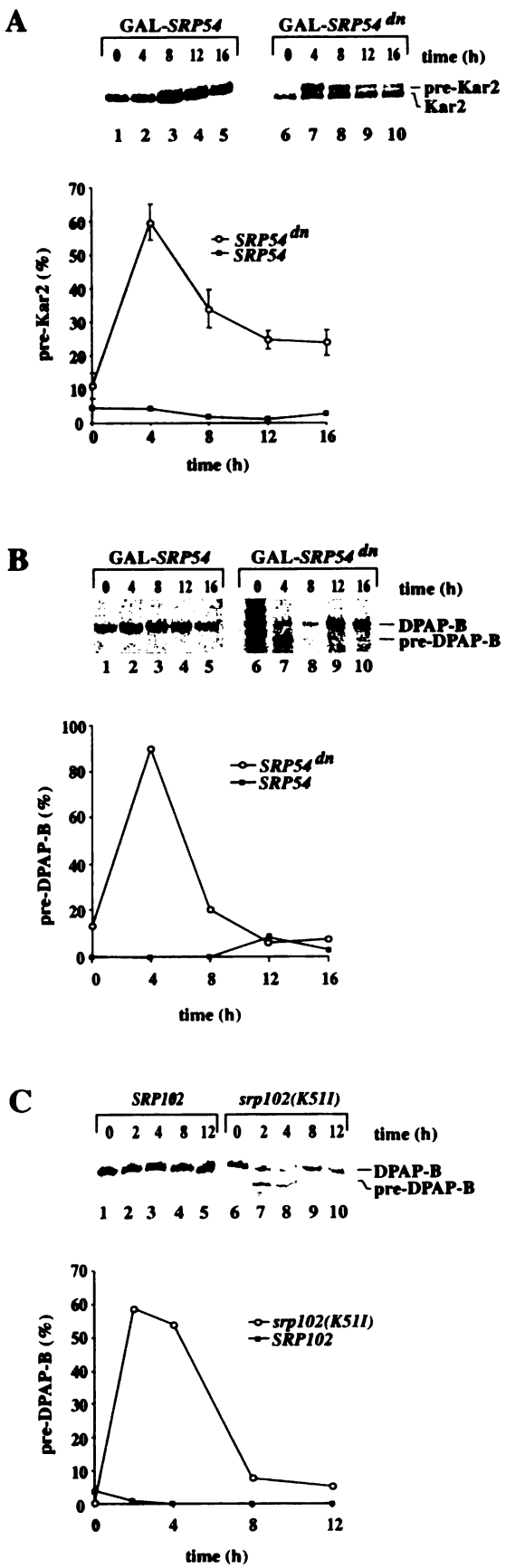
A
GAL-SRP54
GAL-SRP54 dn
0 4 8 12 16
0 4 8 12 16
time (h)
pre-Kar2
Kar2
1 2 3 4 5
6 7 8 9 10
pre-Kar2 (%)
SRP54 dn
SRP54
time (h)
B
GAL-SRP54
GAL-SRP54 dn
0 4 8 12 16
0 4 8 12 16
time (h)
DPAP-B
pre-DPAP-B
1 2 3 4 5
6 7 8 9 10
pre-DPAP-B (%)
SRP54 dn
SRP54
time (h)
C
SRP102
srp102(K511)
0 2 4 8 12
0 2 4 8 12
time (h)
DPAP-B
pre-DPAP-B
1 2 3 4 5
6 7 8 9 10
pre-DPAP-B (%)
srp102(K511)
SRP102
time (h)

Figure 2-1

Figure 2. Adaptation is a reversible, physiological process. Kar2 immunoprecipitation in the *SRP54*^{dn} system. Cells (SMY211) were grown to mid-log phase in raffinose-containing synthetic medium lacking uracil and shifted to galactose-containing medium. After 16 h, the cells were washed free of galactose and returned to raffinose-containing medium for a period of 24 h. After this recovery period, cells were again shifted to galactose-containing medium and allowed to grow for 12 h. Cells were labeled with [^{35}S]-methionine at the indicated time points, and Kar2 was immunoprecipitated as described in Figure1. Cytosolic precursor forms (preKar2) and lumenal forms (Kar2) are indicated. The relative amount of untranslocated protein at each time point was quantified and graphed as in Figure 1A. Differences in protein levels in lane 1, 4, 5, and 6 are due to experimental handling.

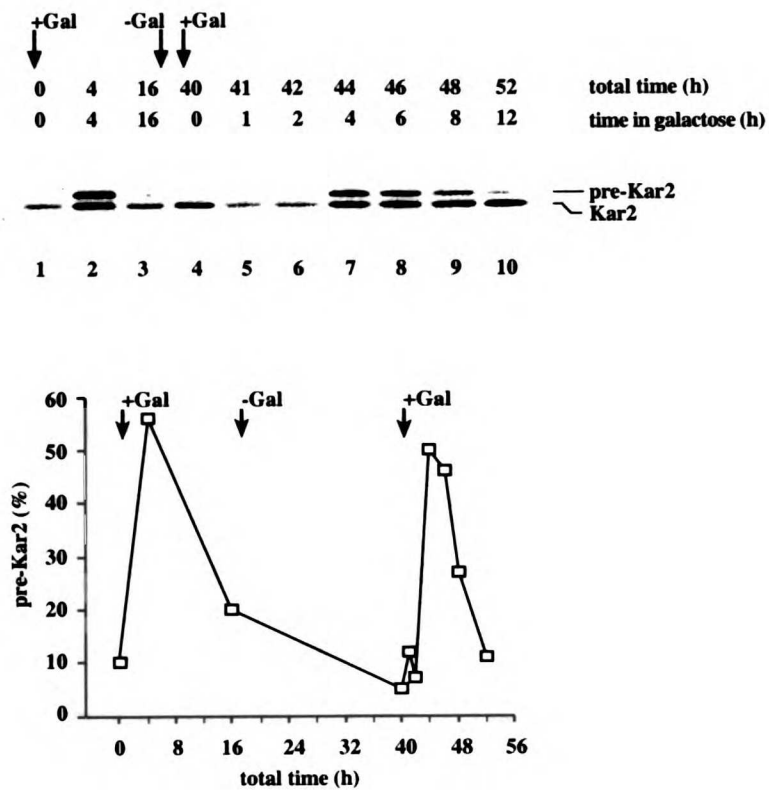
+Gal
-Gal
+Gal
0 4 16 40 41 42 44 46 48 52
total time (h)
0 4 16 0 1 2 4 6 8 12
time in galactose (h)
pre-Kar2
Kar2
1 2 3 4 5 6 7 8 9 10
+Gal
-Gal
+Gal
pre-Kar2 (%)
60
50
40
30
20
10
0
0 8 16 24 32 40 48 56
total time (h)

Figure 2-2

Figure 3. The translocon in adapted cells is identical to that of wildtype cells. Sec63 complex proteins were purified from the following strains: SMY212 (lane 1, no tag); SMY268 bearing pSM131 (lane2, Sec64prA); SMY268 bearing pSM110 (lane 3, Sec63prA); *srp102::URA3*, SMY266 (lane 4 and 5, Sec63prA) as described in Materials and Methods. Cells were grown in raffinose-containing medium to mid-log phase and switched to growth in galactose-containing medium lacking methionine. Cultures were maintained in log phase in galactose-containing medium for 24 h (lanes 1-3). Alternatively, cultures were grown to mid-log phase in YPD at 23°C (lane 4 and 5) and switched to 37°C for 12 h (lane 5). Cells were steady state labeled with [^{35}S]-methionine for 45 min to 1 h at 30°C (lanes 1-3), 23°C (lane 4), or 37°C (lane 5), and membranes were isolated. Digitonin extracts of membranes were incubated with IgG Sepharose for 3 h at 4°C with rotation. The IgG Sepharose beads were washed and protein complexes were eluted with 100 mM glycine pH 2.0. The eluate was concentrated by TCA precipitation and analyzed by SDS-PAGE. The major protein components of the translocon are indicated.

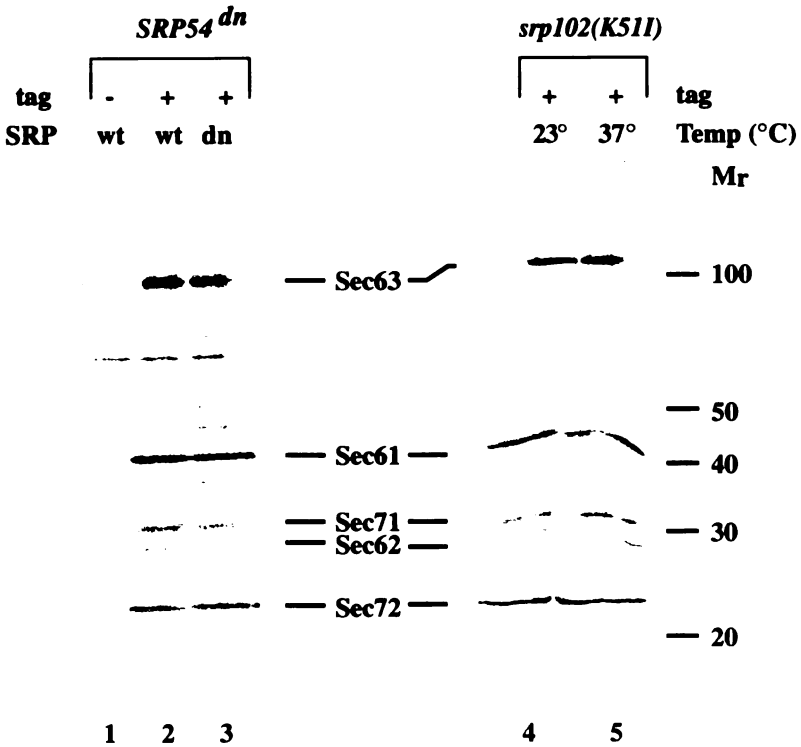
SRP54 dn
srp102(K51I)
tag
SRP
-
+
+
wt
wt
dn
+
+
23°
37°
tag
Temp (°C)
Mr
Sec63
Sec61
Sec71
Sec62
Sec72
100
50
40
30
20
1
2
3
4
5

Figure 2-3

Figure 4. Heat shock and chaperone gene transcription is induced during adaptation. Strains were grown to mid-log phase in either YPD at 23°C ($SR\beta^{ts}$ (SMY288); versus $SR\beta^{wt}$ (SMY286)), or 30°C (ΔSRP54 (SMY284a); ΔSRP102 (YTH119); ΔSCR1 (SOY60); all versus W303 rho$^-$), or in synthetic raffinose-containing medium at 30°C (*SRP54^{dn}* (SMY211); versus *SRP54^{wt}* (SMY212)). The $SR\beta^{ts}$ strain and the $SR\beta^{wt}$ control strain were then both shifted to 37°C for the times indicated. The *SRP54^{dn}* strain and the *SRP54^{wt}* control strain were shifted to galactose-containing medium for the times indicated. It was critical to ensure that all strains were diluted as necessary to keep them continuously in log phase growth, and the medium used was derived from the same batch for each experiment. At the indicated time points, cells were centrifuged and snap-frozen in liquid nitrogen. Fluorescently labeled cDNA probes were made as described in Materials and Methods. Cy3 (green) labeled probes are $SR\beta^{wt}$ at 23°C (0 h time point) or 37°C (2-12 h time points), W303 rho$^-$, and *SRP54^{wt}* in raffinose (0 h time point) or galactose (2-31 h time points). Cy5 (red) labeled probes are $SR\beta^{ts}$, *SRP54^{dn}*, ΔSRP54, ΔSRP102, and ΔSCR1. For each time point or deletion, the differentially labeled probe pairs were mixed and hybridized to a microarray, and the relative abundance of each mRNA was measured by intensity of red or green fluorescence. The red/green fluorescence intensity ratio gives a measure of relative expression for each ORF as shown in the color scale. Brightest red color blocks indicate genes most highly induced relative to the control strain, brightest green blocks represent highest repression, black indicates no change in expression, and gray indicates no data. (A)

Relative expression levels of selected genes encoding for heat shock proteins and chaperones are depicted with color blocks. (B) Relative expression levels of several heat shock and chaperone genes throughout the $SR\beta^{ts}$ time course. (C) Relative expression levels of several heat shock and chaperone genes throughout the $SRP54^{dn}$ time course.

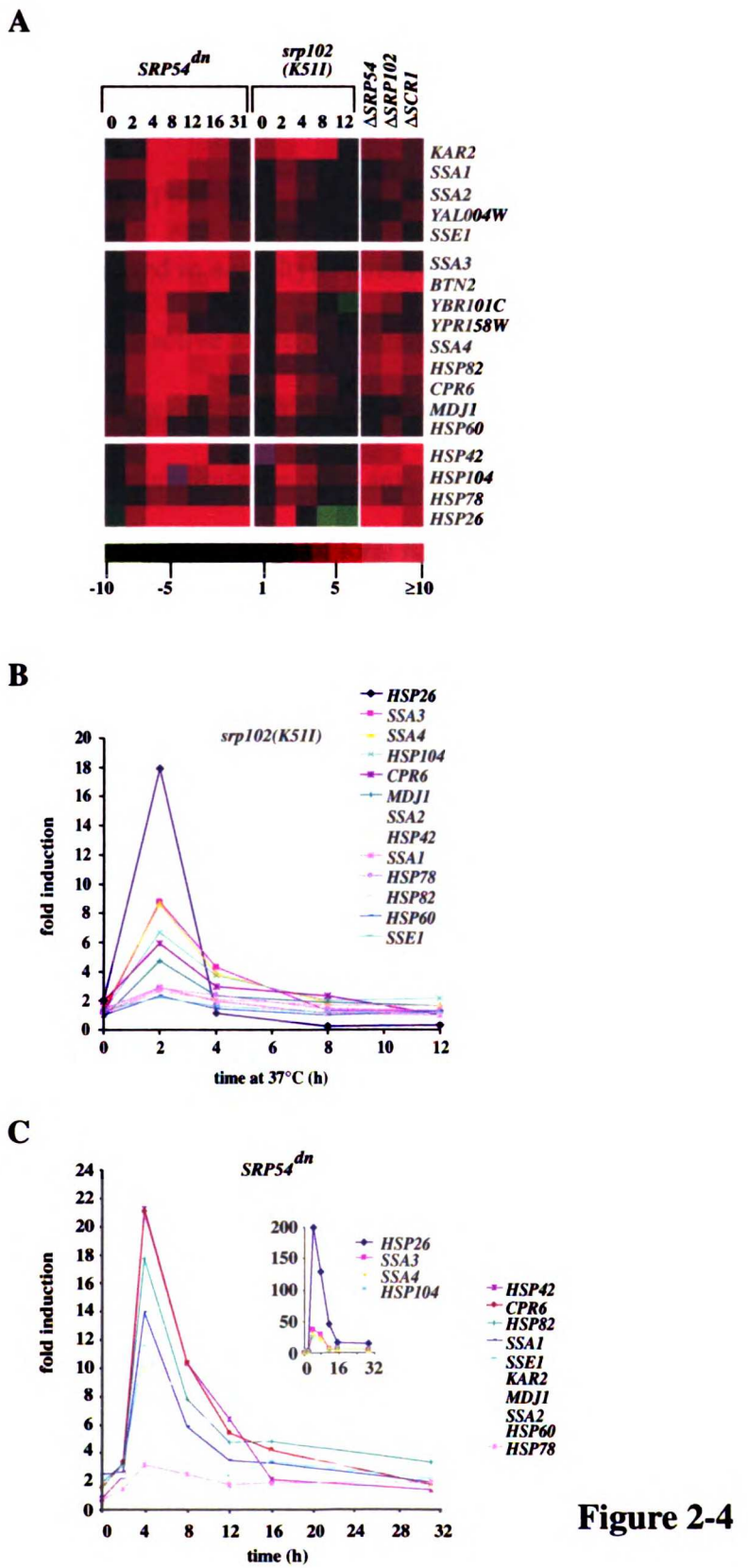

Figure 2-4

Figure 5. Constitutive high expression of heat shock proteins and chaperones is not sufficient to adapt to the loss of SRP54. Accumulation of untranslocated Kar2 upon induction of *SRP54dn* was measured in a wildtype strain (SMY211; open boxes; n=4) and in a strain containing a constitutively active allele of HSF1 (SMY226 ($HSF1^{C}$; *hsf::LEU2*, with pHF35); filled boxes; n=2). Cells were grown in galactose-containing medium for 4 h, labeled and processed for immunoprecipitation with anti-Kar2 as described in Figure. 1. The precursor form of Kar2 is plotted as percentage of total Kar2 immunoprecipitated.

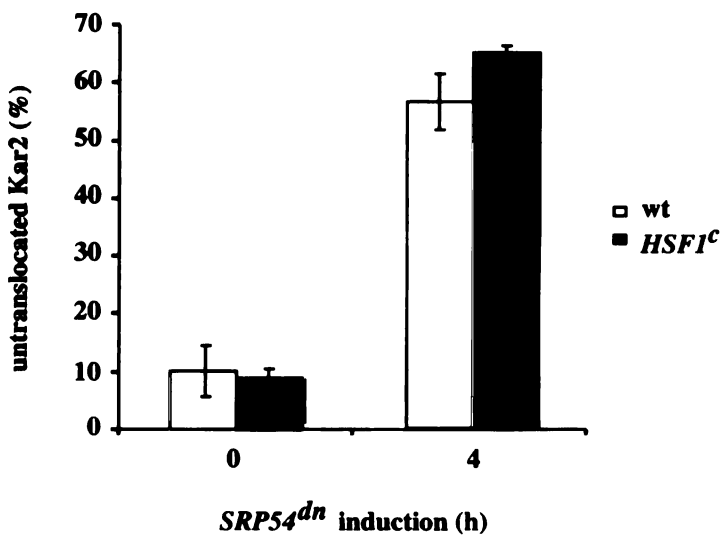
70
60
50
40
30
20
10
0
untranslocated Kar2 (%)
0
4
SRP54dn induction (h)
wt
HSF1c

Figure 2-5

Figure 6. Ribosome biogenesis is repressed during adaptation. Strains, conditions, and color codes are exactly as described in Figure 4. (A) Relative expression levels of selected genes involved in ribosome biogenesis are depicted. (B) Relative expression levels of several ribosomal protein genes throughout the SRβ^{ts} time course are plotted.

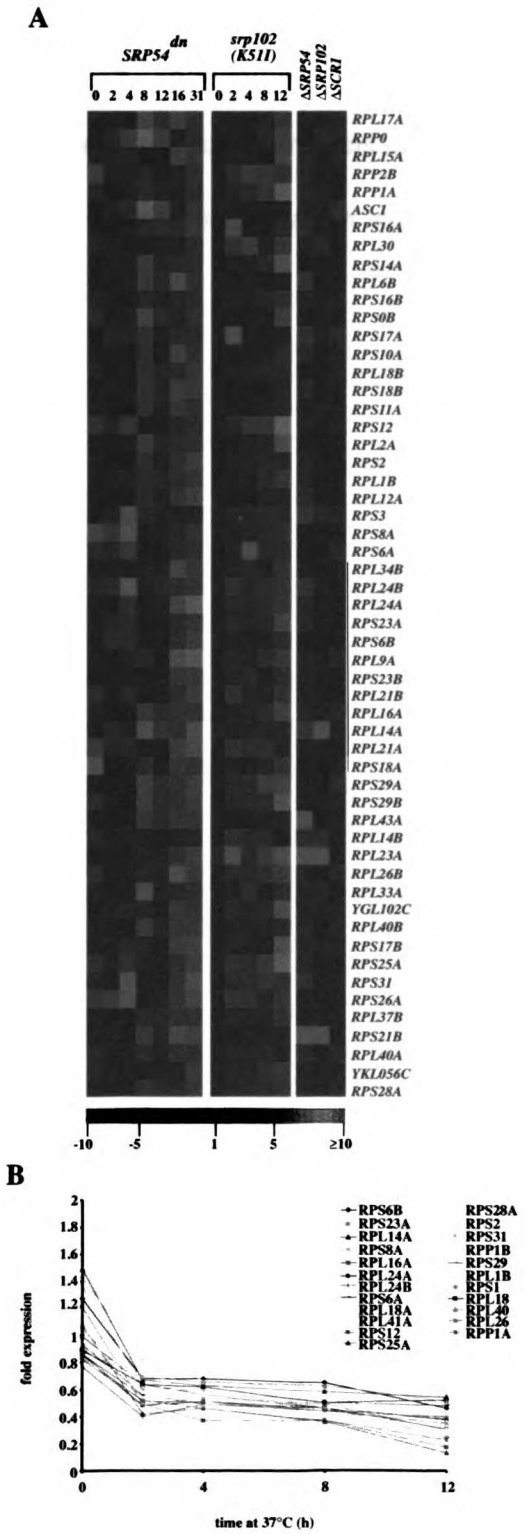
A
SRP54 dn
srp102 (K511)
ΔSRP54 ΔSRP102 ΔSCR1
0 2 4 8 12 16 31
0 2 4 8 12
RPL17A
RPP0
RPL15A
RPP2B
RPP1A
ASC1
RPS16A
RPL30
RPS14A
RPL6B
RPS16B
RPS0B
RPS17A
RPS10A
RPL18B
RPS18B
RPS11A
RPS12
RPL2A
RPS2
RPL1B
RPL12A
RPS3
RPS8A
RPS6A
RPL34B
RPL24B
RPL24A
RPS23A
RPS6B
RPL9A
RPS23B
RPL21B
RPL16A
RPL14A
RPL21A
RPS18A
RPS29A
RPS29B
RPL43A
RPL14B
RPL23A
RPL26B
RPL33A
YGL102C
RPL40B
RPS17B
RPS25A
RPS31
RPS26A
RPL37B
RPS21B
RPL40A
YKL056C
RPS28A
-10 -5 1 5 ≥10
B
fold expression
time at 37°C (h)
RPS6B
RPS23A
RPL14A
RPS8A
RPL16A
RPL24A
RPL24B
RPS6A
RPL18A
RPL41A
RPS12
RPS25A
RPS28A
RPS2
RPS31
RPP1B
RPS29
RPL1B
RPS1
RPL18
RPL40
RPL26
RPP1A

Figure 2- 6

Figure 7. Decreased protein synthesis caused by cycloheximide treatment can suppress translocation defects due to *SRP54^{dn}* expression. *SRP54^{dn}* cells were grown in galactose for 3 h, and cycloheximide was added to the concentrations indicated for an additional hour. Cells were labeled and processed for immunoprecipitation with anti-Kar2 as described in Figure 1.

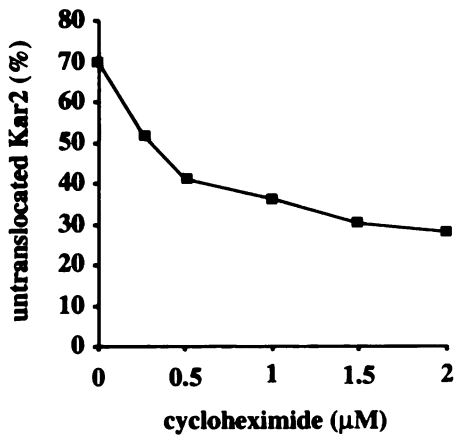
untranslocated Kar2 (%)
80
70
60
50
40
30
20
10
0
0
0.5
1
1.5
2
cycloheximide (μM)

Figure 2-7

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CHAPTER III:

Transcriptional differences between the loss of SRP, the SRP receptor, or the SRP RNA

INTRODUCTION

Although there are many levels at which biological processes may be regulated, a change in a cell's status or growth conditions is frequently accompanied by changes in mRNA levels for multiple genes. Until recently, techniques to measure expression changes of genes were restricted to methods such as northern blotting which have the limitation of measuring only one mRNA at a time. While this work was in progress, the entire sequence of the *Saccharomyces cerevisiae* genome was completed (see Cherry et al., 1997) for physical and genetic maps and (http://genome-www.stanford.edu/Saccharomyces/). This advance quickly led to the development of high-density DNA microarray technologies for expression monitoring (Chee et al., 1996; Lashkari et al., 1997; Lockhart et al., 1996; Schena et al., 1995), providing a relatively simple method of rapidly assaying gene expression changes on a global level.

The DNA microarrays most readily available and cost efficient for the academic lab consist of DNA sequences representing all known yeast open reading frames (ORFs) printed onto glass slides with a robotic printing device. The ORFs are generated by amplification by PCR using a set of primer pairs that is commercially available (DeRisi et al., 1997). However, the technology is continually evolving, and the development of long (~70mers) oligonucleotide arrays is in progress. This technology would have numerous advantages over the current DNA microarrays including freedom from cumbersome and sometimes inefficient PCR amplification and the ability to choose regions within ORFs that minimize cross-hybridization potential. The ongoing development of high-throughput, multi-channel

oligonucleotide synthesizers will help to make this technology a viable and attractive alternative.

Genome-wide expression analysis produces an immense amount of data, and the challenge becomes how to begin analyzing it. Initially, analysis is often limited to those genes that are most extremely induced or repressed, and a vast amount of insight can be gained in this way. However, it is becoming clear that low-amplitude regulatory profiles are common, biologically significant expression patterns (Hughes et al., 2000), and co-regulation is frequently observed for genes with similar functions (Eisen et al., 1998). This emphasizes the need for tools to order genes with similar patterns of expression, and programs have been developed that cluster gene expression measurements in multiple experiments by standard statistical methods. The results can be displayed graphically, allowing the user to more easily infer from gene expression changes the status of cellular processes (Eisen et al., 1998). Using large numbers of expression profile datasets for clustering improves the organization to the point that the function of unknown ORFs can often be inferred from the groupings (Hughes et al., 2000). The same cluster analysis can also be performed for whole experiments (array clustering) to determine similarity between different strains or conditions.

The development of DNA microarray technology was particularly useful for investigating the cellular response to the loss of the SRP protein-targeting pathway. We lacked a genetic handle on the adaptation process that would allow us to perform traditional screen for components involved in the response. Furthermore, it became clear that this adaptive response is most likely not due to a small number of changes in the cell but rather is highly complicated and consists of multiple parts. Because of this, DNA microarrays were particularly well suited for aiding our understanding of this problem on a more global level.

In Chapter 2, we looked at two major programs that were similar in both inducible SRP disruption systems. Here, we expand our discussion of genomic data to include differences between deletion strains as well as differences between inducible SRP pathway mutants.

MATERIALS AND METHODS

Strains Used in this Study

W303 (*MATα, leu2-3-112, his3-11, trp1-1, ura3-1, can1-100, ade2-1);* SMY246 (*MATα, leu2-3-112, his3-11, trp1-1, ura3-1, can1-100, ade2-1, rho*$^{-}$); YTH119 (W303, *MATα, srp102::URA3*) from T. Hu, University of California, San Francisco; SMY284a (W303 *MATa, srp54::neo*) from Gustavo Pesce, University of California, San Francisco; SOY60 (W303, *MATα, scr1::HIS3*) (Ogg et al., 1992); SMY211 (W303, pDN66); SMY212 (W303, pGal*SRP54*); SMY286 (YTH119, pTH123), SMY288 (YTH119, pSO462).

Plasmids used in this study

pGalSRP54 (Gal-*SRP54*, URA3, CEN4/ARS1) (Hann and Walter, 1991); pDN66 (Chapter 2); pTH123 (*SRP102*-3xFlag, TRP1, CEN6/ARSH4) from Dr. T. Hu, University of California, San Francisco; pSO462 (*srp102*(K51I)-HA, TRP1, CEN6/ARSH4) (Ogg et al., 1998).

Genomic Arrays: Sample Preparation and Hybridization

Experimental procedures are exactly as described in Chapter 2. The reference samples were grown in the same media and temperature conditions in order to cancel out many differences due to growth conditions. The galactose inducible *SRP54*^{dn} cells were compared to galactose

inducible *SRP54*^{wt} cells to control for both the carbon source shift and protein overexpression. Similarly, the SRβ^{ts} cells were compared to SRβ^{wt} cells also grown at 37°C to control for the temperature shift. SRP deletion strains were compared to rho^0 strains to eliminate variance due to the rho$^-$ phenotype of all SRP deletions.

Genomic Data Analysis and Categorization

Microarrays were visualized using a GenePix scanner (Axon), and fold changes in mRNA levels relative to control samples were determined using GenePix analysis software. Cluster analysis was performed as described (Eisen et al., 1998) and visualized with Treeview software (download at http://rana.Stanford.EDU/software/). Our experiments were clustered with published data sets (Chu et al., 1998; DeRisi et al., 1997; Spellman et al., 1998; Travers, 2000), unpublished data sets from our laboratory, and unpublished data sets kindly provided by Joe DeRisi (University of California, San Francisco, CA) and Pat Brown (Stanford University, Palo Alto, CA).

Scatterplots

Scatterplots and regression analysis were made using Microsoft Excel. Red/green expression ratios in each set of SRP deletion experiments were converted to $\log_{10}$ values and plotted for each ORF. A line of best fit for the scatterplot and confidence intervals = ±3 SD of the mean were drawn. ORFs residing outside the confidence intervals were chosen and grouped according to specificity for SRP pathway deletion.

Pulse-chase experiments and Immunoprecipitation

Strains to be labeled were induced as described in Chapter 2. *SRP54*^{dn} cells were grown and labeled at 30°C and *srp102*(K51I) cells were grown and labeled at 37°C. At 30 min prior to the time point, cells were harvested and washed two times in prewarmed media lacking methionine. Cells were resuspended at 3 OD_{600}U/ml and grown for the remainder of the time point (approximately 10 min). 20 μCi/OD ProMix (Amersham) was added for 10 min and chased by addition of 2 mM each methionine and cysteine. For the SRβ^{ts} cells, 200 μM cycloheximide was also added. At 0, 4, 8, 16, and 32 min, 1.5 ml aliquots were removed to chilled tubes containing 5 μl 3 M sodium azide and 15 μl 5x YP, mixed and quick-frozen in liquid nitrogen. Extracts and immunoprecipitations were performed as described in Chapter 2. Quantitation was performed with a Molecular Dynamics (Sunnyvale, CA) Storm 840 imager and ImageQuant Software.

RESULTS

SRP pathway deletion strains

The SRP pathway deletion strains tested (SRP54, SRβ, and SCR1) are, for the most part, very similar. For example, in array clustering analysis performed with over 200 datasets, these three deletion strains cluster together (Figure 3-2, SRP deletions). However, when comparing expression of individual ORFs between the strains, there are some differences in transcriptional responses observed, and it is possible that some of these responses may indicate relationships between the function of the gene product and the SRP component that is deleted. ORFs that show transcriptional responses specific to one of the three SRP deletions will be listed here. However, caution is urged in interpreting these differences as

each experiment was done only once. As we have seen both empirically and from other work (Hughes et al., 2000), many ORFs can vary more than two-fold even in comparisons of wildtype strains. This emphasizes the need for microarray experiments of single strain comparisons to be done several times with data points compared to determine which ORF differences are significant and reproducible.

With this caveat in mind, we proceeded to look for differences between different SRP pathway deletions. In order to determine significant outliers in experiment pairs, scatterplots were made comparing $\log_{10}$ R/G ratios in each set of SRP deletion experiments. A line of best fit was calculated and confidence intervals = ±3 SD of the mean were drawn (Figures 3-1A, B, and C). ORFs residing outside the confidence intervals were chosen and grouped according to specificity for SRP pathway deletion. Those ORFs are summarized in Figure 3-6.

Differences between SRP54^{dn} *and* SRβ^{ts} *time courses*

Cluster analysis demonstrates that, although a large number of expression patterns for ORFs are similar between the *SRP54^{dn}* and SRβ^{ts} time courses, many clusters and individual ORFs have distinct expression profiles. Furthermore, when array clustering the time course experiments with a large number of other datasets, not all time points from different induction systems cluster together. The 2-12 h time points of the *SRP54^{dn}* time course cluster together (Figure 3-2, *SRP54^{dn}*), but separately from the 16 h and 31 h time points. These two experiments cluster together with the 2-12 h time points of the SRβ^{ts} time course (Figure 3-2, SRβ^{ts}-*54^{dn}*). Therefore, as strains are grown longer to allow full adaptation, they appear to become more similar with regard to mRNA expression patterns. This divergence between

the two inducible SRP disruption systems may indicate that, in response to the loss of the SRP pathway, different stress responses may occur depending on growth conditions.

14 clusters were identified by visual inspection to have different patterns of expression between the two inducible SRP pathway systems. Strikingly, in each case, upregulation was observed in the *SRP54*^{dn} system but not in the SRβ^{ts} system. For some of these clusters, this may be due to the fact that the SRβ^{ts} strain is normalized to a heat shocked control strain in which these genes are highly upregulated (see below). This could potentially mask significant upregulation occurring due to the loss of SRβ function. For both time courses, it would be useful to repeat the genomics experiments by internally comparing each time point to the uninduced point to supplement the experiments already performed.

Identified clusters that differ between SRP disruption systems are shown in Figure 3-3A and B. The cluster groups include:

- **proteasome genes** (induced strongly, 2-8 h time points)
- **members of the short-chain alcohol dehydrogenase (AAD) family of genes** (strongly induced in late time points of *SRP54*^{dn} system; strongly repressed in late time points of SRβ^{ts} system)
- **genes involved in glycolysis** (generally induced throughout time course)
- **chromatin structure/histone genes** (induced 2-12 h time points)
- **mitochondrial/energy generation genes** (induced 2-12 h time points, then repressed)
- **secretion** (induced 2-12 h time points)
- **methionine biosynthesis/amino acid metabolism** (induced throughout time course)
- **subtelomeric genes** (induced throughout time course)

- **elongation factors (includes NAC subunit)** (induced until latest time points, then repressed)
- **unknown ORFs**

Of the displayed clusters, those which demonstrate upregulation in heat shock experiments include the glycolysis, mitochondrial/energy generation, subtelomeric genes, aging, and proteasome clusters (Audrey Gasch and Pat Brown, unpublished datasets), and this could potentially explain some of the differences observed between the *SRP54*^{dn} and SRβ^{ts} systems. However, differences in the other displayed clusters, including AAD, histones, methionine biosynthesis, secretion, and elongation factor clusters, cannot be explained in this way.

We have begun to analyze some of the differences observed between the two inducible SRP disruption systems. For example, *SEC71* is a member of the small cluster of secretion related genes upregulated early in the *SRP54*^{dn} time course. However, we have tested the effect of deletion of genes encoding a number of ER membrane proteins (*SEC71*, *SEC72*, and *SSH1*) on the ability of a cell to adapt, and see little or no effect (Figure 3-4). Thus, upregulation of these genes cannot be required for adaptation, and the reason for differences in some secretion genes remains unclear.

We have also examined the role of protein degradation in adaptation to the loss of the SRP pathway as briefly described in Chapter 2. These unpublished results are shown here in more detail. We used pulse-chase experiments to determine the half-lives of untranslocated proteins at early and late time points after SRP pathway disruption. After temperature shift of SRβ^{ts} cells, pre-Kar2 had similar half-lives throughout the adaptation time course ($T_{1/2}$ = 89 min at 2 h; 94 min at 12 after temperature shift) (Figure 3-5A, squares), suggesting that increased degradation of precursor proteins is not responsible for the apparent improvement

of translocation efficiency observed in these cells. Furthermore, the rate of disappearance of precursor was closely mirrored by the rate of appearance of mature protein (Figure 3-5A, circles), suggesting that the majority of precursor disappearance was accounted for by translocation rather than degradation.

In contrast, upon induction of *SRP54^{dn}*, we observed an increased rate of pre-Kar2 disappearance ($T_{1/2}$ = 40 min at 4 h; 23 min at 16 after temperature shift) (Figure 3-5B, squares) that was not accompanied by a corresponding increase in translocated protein (Figure 3-5B, circles), i.e., could not be accounted for by post-translational protein translocation. This analysis involves assumptions that may or may not be true. For example, we have assumed that mature Kar2 is equally stable at different points of induction, but this remains to be experimentally confirmed. Nevertheless, the data suggest that pre-Kar2 is degraded at an increased rate in the *SRP54^{dn}* adapted cells indicating that there may be different ways in which cells can cope with SRP depending on growth conditions or other factors.

DISCUSSION

In this chapter, we have examined some of the differences in expression profiles for various methods of disrupting the SRP protein-targeting pathway. We found several differences between three SRP pathway deletion strains, although the genomic experiments will need to be repeated before these avenues should be examined. The SRP54 specific genes include *YKL071W*. The protein encoded by this gene has similarity to the short-chain alcohol dehydrogenase (AAD) family of proteins (Costanzo et al., 2000) and is regulated by Yap1p

(DeRisi et al., 1997). As discussed above, several members of this family are also found to be upregulated in the *SRP54*^{dn} system but not the SRβ^{ts} system. Due to the high degree of homology between AAD family members (77-97% nucleotide sequence identity), northern blotting experiments using more specific hybridization probes will be required to determine if indeed the entire family is upregulated or if the majority of the signal is due to cross-hybridization. Other genes in this *SRP54* specific group include *YLR041W*, an unknown ORF, and *APG7*, which encodes an enzyme involved in autophagy and possibly cytoplasm-to-vacuole protein targeting (Scott et al., 1996).

The SRβ specific genes that are repressed include *SCW11*, a putative cell wall protein. When this gene is deleted, mutants cannot separate well after division, a phenotype observed in ΔSRP54 strains. Induced genes include *TOR2*, a kinase involved in ribosomal protein gene induction in response to changes of nutrient conditions (Dennis et al., 1999), and *FUN30*, encoding a nuclear protein containing a DNA-dependent ATPase domain (http://www.proteome.com, (Costanzo et al., 2000)).

The SCR1 specific genes that are repressed include two genes involved in cell wall maintenance (*ECM11* and *ECM18*), two transcription factors (*RMS1* and *YHR207C*), and other unknown ORFs. Of the induced genes, one interesting candidate is found in *SEN1*. This gene encodes a DEAD-box type RNA helicase involved in tRNA-splicing as well as snRNA and snoRNA maturation (http://www.proteome.com, (Costanzo et al., 2000)).

While we observed many similarities in expression profiles for the *SRP54*^{dn} and SRβ^{ts} inducible systems, there are also a variety of differences throughout the time courses. These differences may merely be due to the differing growth conditions needed for each inducible system, or they may be more meaningful. It is possible that multiple paths can be taken

along the road to survival of the loss of the SRP pathway. We have already observed that adaptation is a multifaceted response and that adaptation can occur in strains mutated or deleted for components that would seem to be involved in the process (for example HSF mutants, individual chaperones, and deletions of ER membrane proteins). Because adaptation is a physiological response composed of several different stress pathways, perhaps enough redundancy exists in these stress responses that no one pathway is absolutely required. While it remains possible that an "adaptation" specific stress pathway will be found hidden among the numerous uncharacterized ORFs not yet examined, it appears that the most likely scenario for the adaptation to the loss of the SRP protein targeting pathway involves a combination of multiple, common stress pathways.

FIGURE LEGENDS

Figure 3-1: Scatterplots show outliers between SRP deletion strains. Log_{10}-transformed expression ratios for each ORF were plotted for each pair of the three SRP deletion strains examined. A linear regression line was calculated, and confidence intervals representing ±3 SD were drawn. ORFs with expression ratios causing them to fall outside the confidence intervals were identified for later grouping and analysis. SRP strain pairs include: ΔSRP54 vs. ΔSRβ (A), ΔSRP54 v. ΔSCR1 (B), and ΔSCR1 v. ΔSRβ (C).

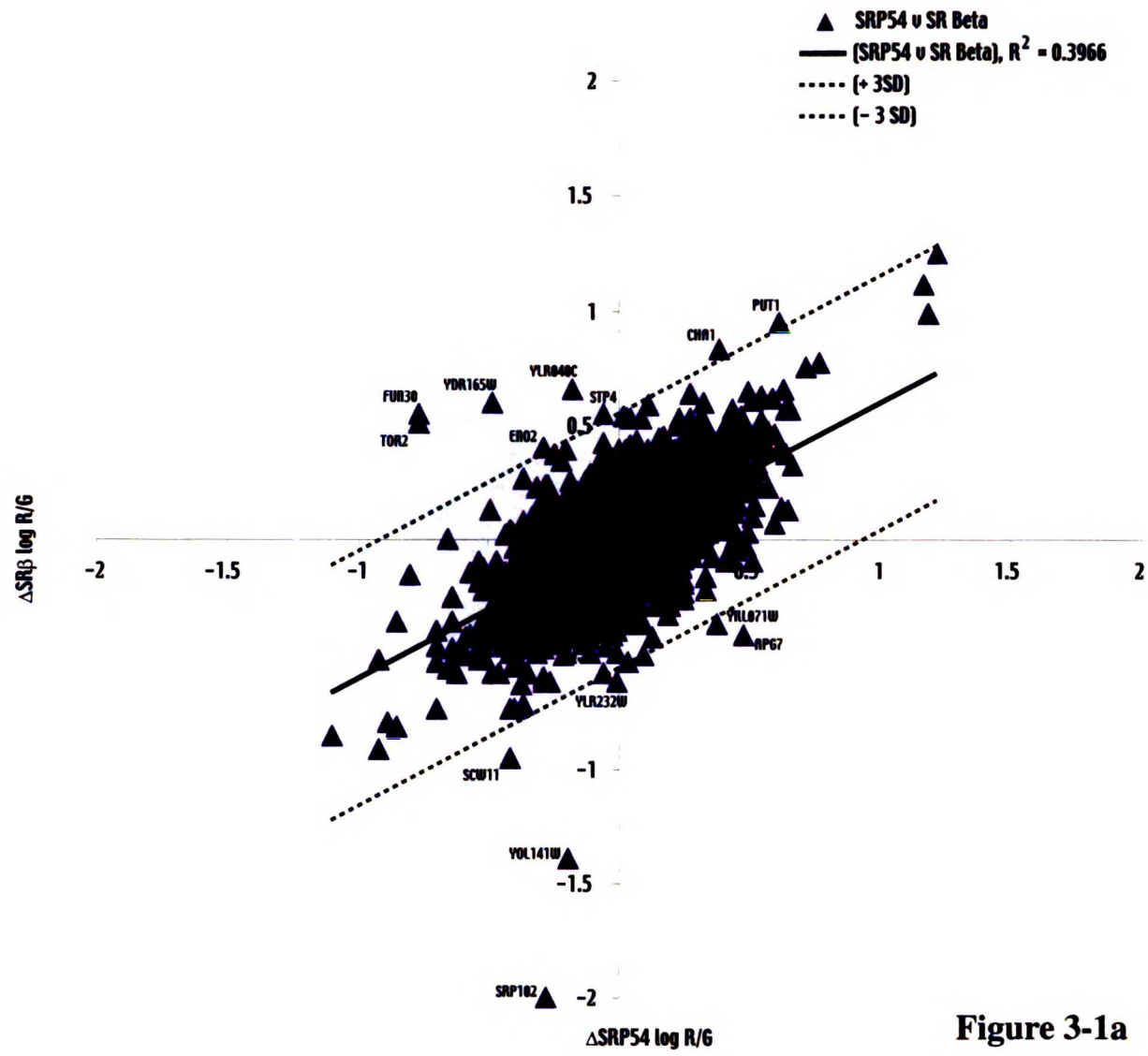

SRP54 v SR Beta
(SRP54 v SR Beta), R2 = 0.3966
(+ 3SD)
(- 3 SD)
ΔSRβ log R/6
ΔSRP54 log R/6
PUT1
CHA1
YLR040C
YDR165W
FUR30
STP4
TOR2
ENO2
YHL071W
RPG7
YLR232W
SCW11
YOL141W
SRP102

Figure 3-1a

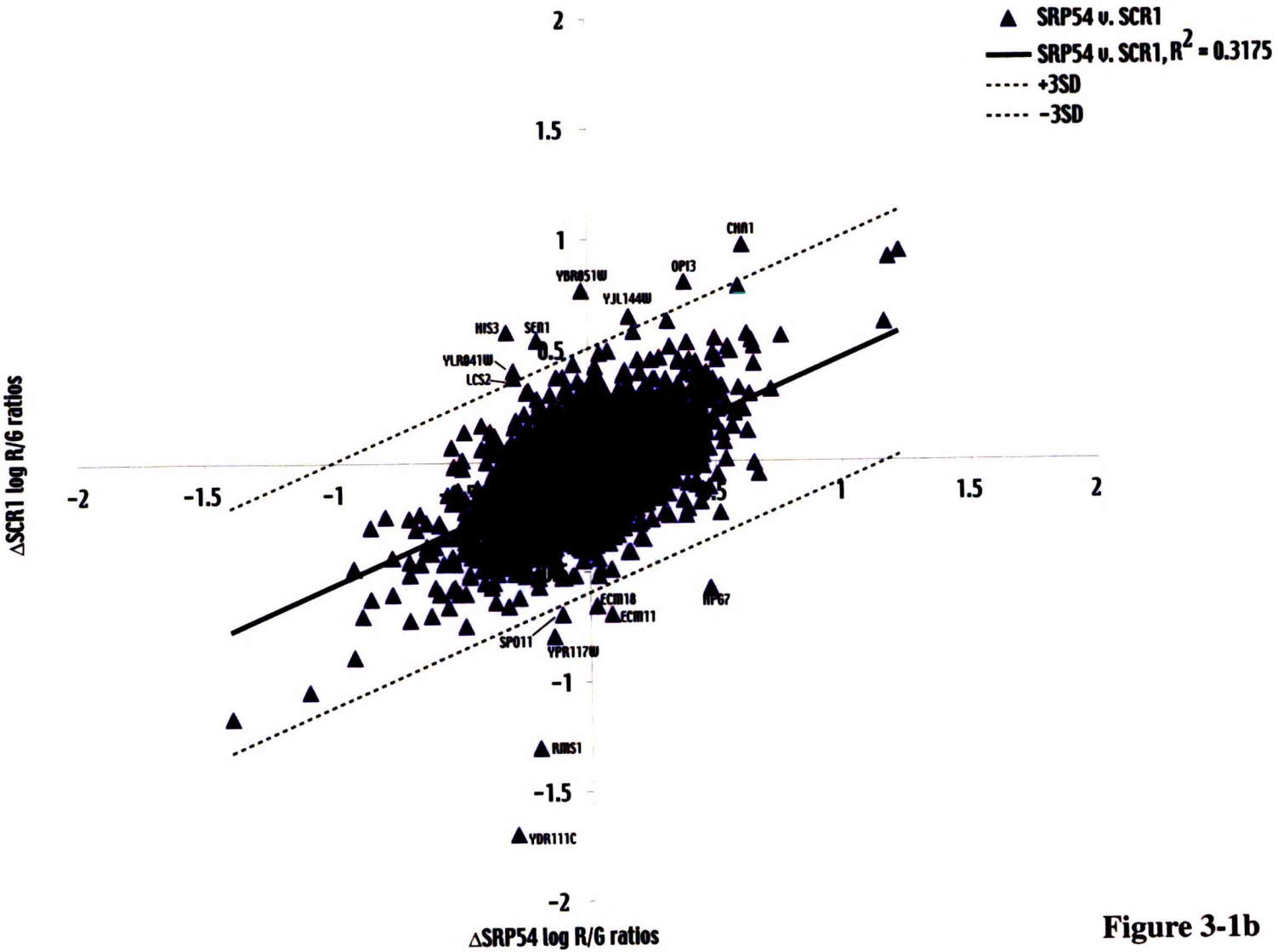

SRP54 v. SCR1
SRP54 v. SCR1, R2 = 0.3175
+3SD
-3SD
2
1.5
1
0.5
-1
-1.5
-2
-2
-1.5
-1
1
1.5
2
CHA1
OPI3
YBR051W
YJL144W
HIS3
SER1
YLR041W
LCS2
ECM18
ECM11
HPG7
SPO11
YPR117W
RIM51
YDR111C
ΔSCR1 log R/G ratios
ΔSRP54 log R/G ratios

Figure 3-1b

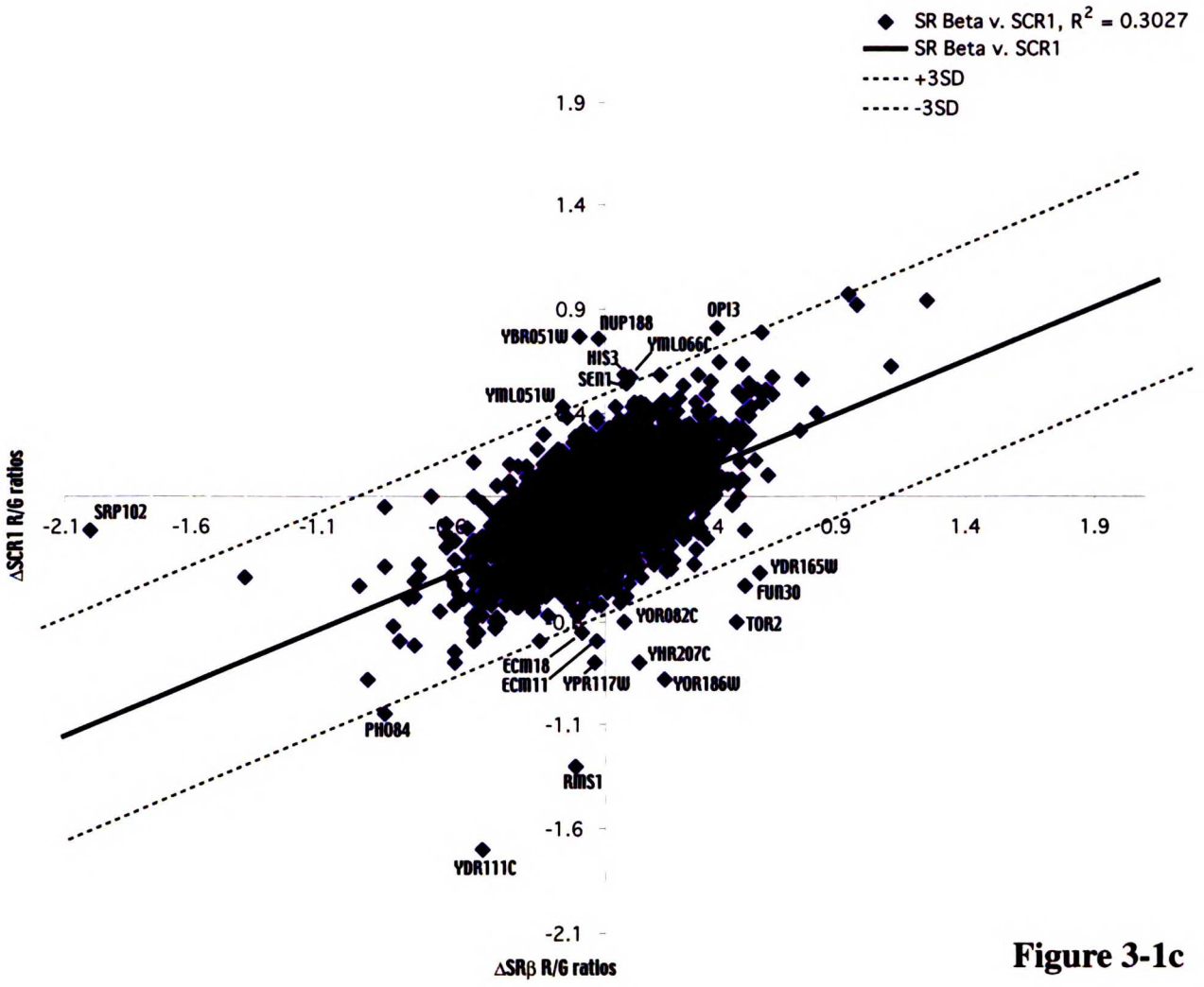

SR Beta v. SCR1, R2 = 0.3027
SR Beta v. SCR1
+3SD
-3SD
ΔSCR1 R/6 ratios
ΔSRβ R/6 ratios
1.9
1.4
0.9
-1.1
-1.6
-2.1
-1.6
-1.1
0.9
1.4
1.9
SRP102
YBR051W
NUP188
OPI3
YML066C
HIS3
SEN1
YML051W
YDR165W
FUR30
TOR2
YOR082C
YHR207C
YOR186W
YPR117W
ECM18
ECM11
PHO84
RIM51
YDR111C

Figure 3-1c

Figure 3-2: Datasets representing over 200 stress conditions were array clustered. The data displayed are one continuous array cluster reading from left to right and top to bottom. Experiment groups are marked in color and named, and SRP related experiments are marked by a red line. The early time points of the *SRP54*^{dn} cluster separately from the remaining SRP experiments. Deletion strains cluster tightly together and in the same large cluster group as the remaining *SRP54*^{dn} late time points and the SRβ time points.

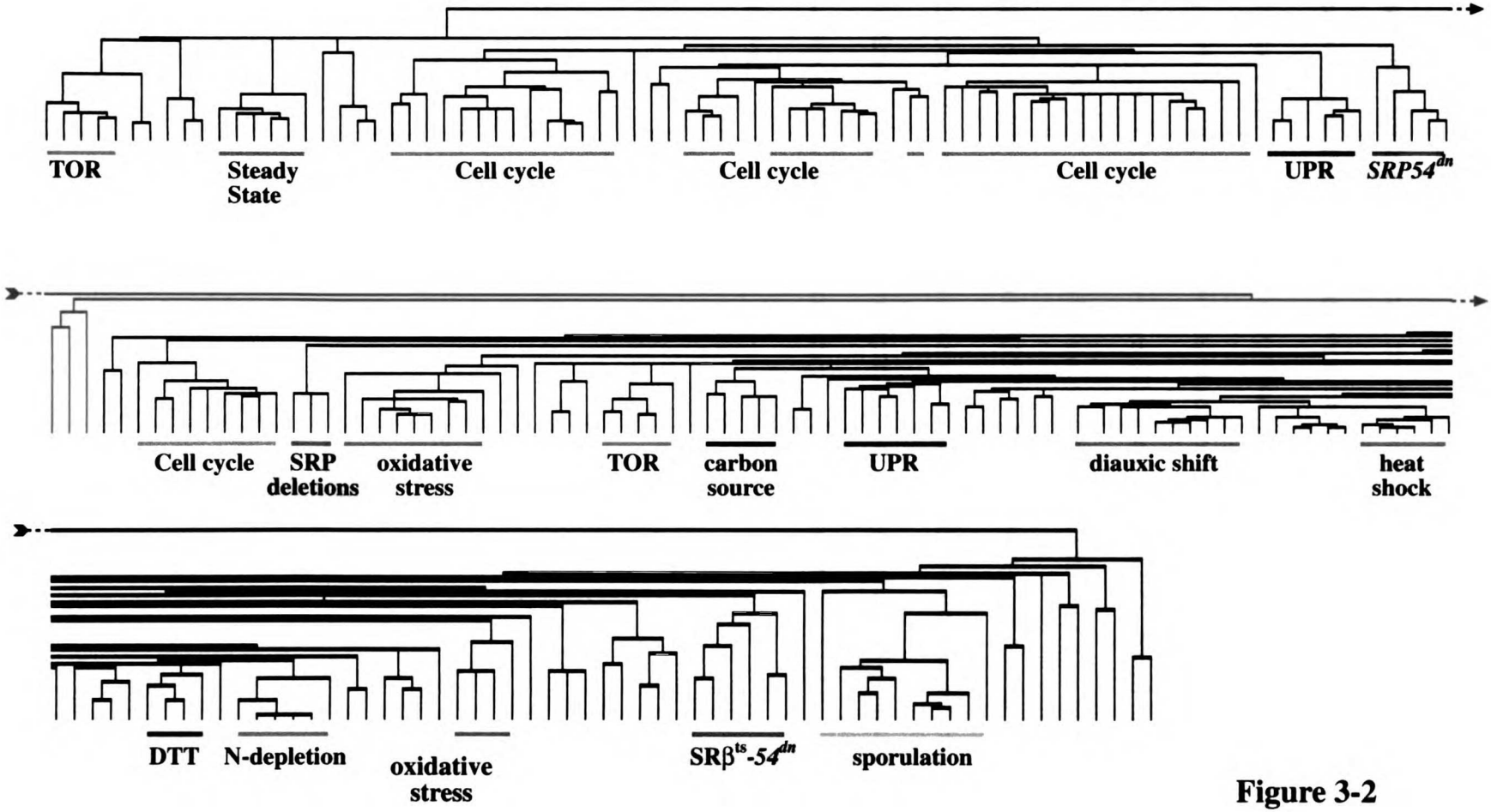


Figure 3-2

Figure 3-3: Datasets representing over 200 stress conditions including *SRP54*^{dn} time course and a SRβ time course were clustered by gene expression ratios. 14 clusters were identified by visual inspection to have different patterns of expression between the two inducible SRP pathway systems. In each cluster, upregulation was observed in the *SRP54*^{dn} system but not in the SRβ^{ts} system. Cy3 (green) labeled probes are SRβ^{wt} at 23°C (0 h time point) or 37°C (2-12 h time points), and *SRP54*^{wt} in raffinose (0 h time point) or galactose (2-31 h time points). Cy5 (red) labeled probes are SRβ^{ts} and *SRP54*^{dn}. Brightest red color blocks indicate genes most highly induced relative to the control strain, brightest green blocks represent highest repression, black indicates no change in expression, and gray indicates no data. (* represents an unknown cluster which is a part of a larger cluster also containing the proteasome cluster shown).

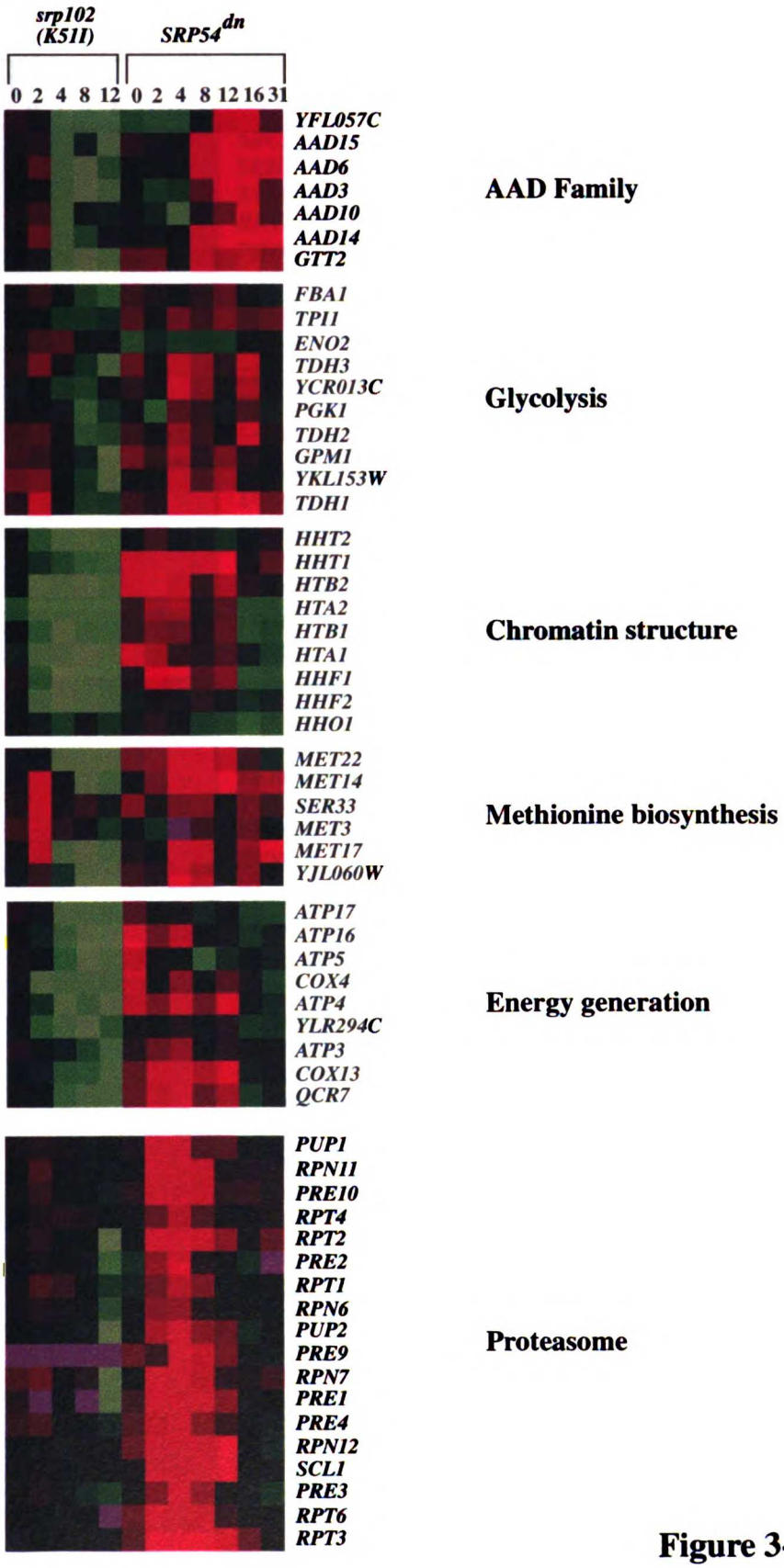
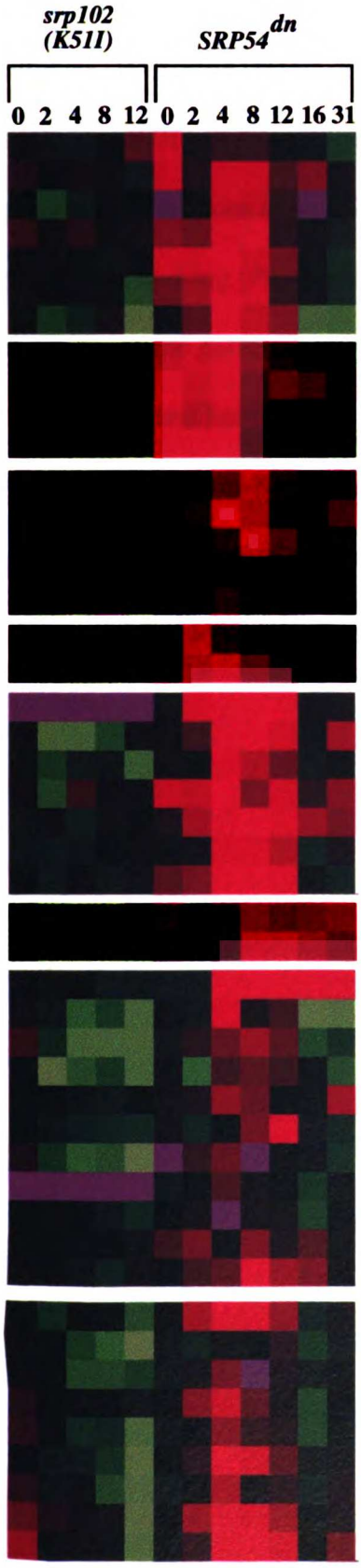

srp102 (K51I)
SRP54dn
0 2 4 8 12 0 2 4 8 12 16 31
YFL057C
AAD15
AAD6
AAD3
AAD10
AAD14
GTT2
AAD Family
FBA1
TPI1
ENO2
TDH3
YCR013C
PGK1
TDH2
GPM1
YKL153W
TDH1
Glycolysis
HHT2
HHT1
HTB2
HTA2
HTB1
HTA1
HHF1
HHF2
HHO1
Chromatin structure
MET22
MET14
SER33
MET3
MET17
YJL060W
Methionine biosynthesis
ATP17
ATP16
ATP5
COX4
ATP4
YLR294C
ATP3
COX13
QCR7
Energy generation
PUP1
RPN11
PRE10
RPT4
RPT2
PRE2
RPT1
RPN6
PUP2
PRE9
RPN7
PRE1
PRE4
RPN12
SCL1
PRE3
RPT6
RPT3
Proteasome

Figure 3-3a



Secretion

Elongation factors

Subtelomeric

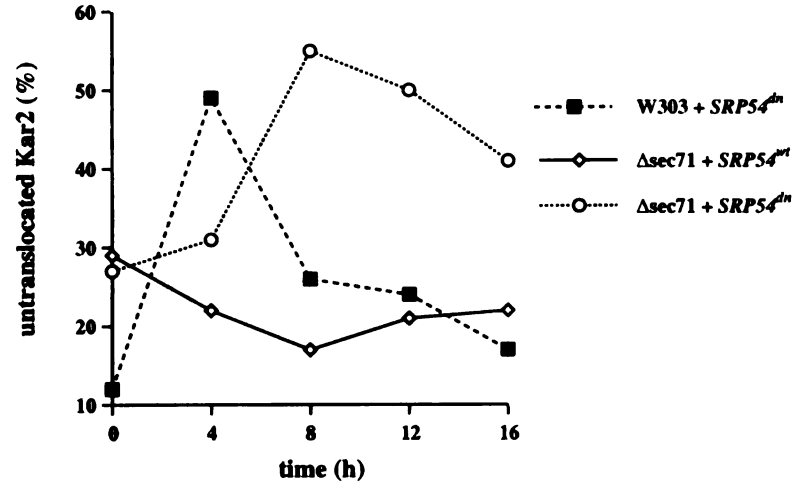
Aging/Protein degradation

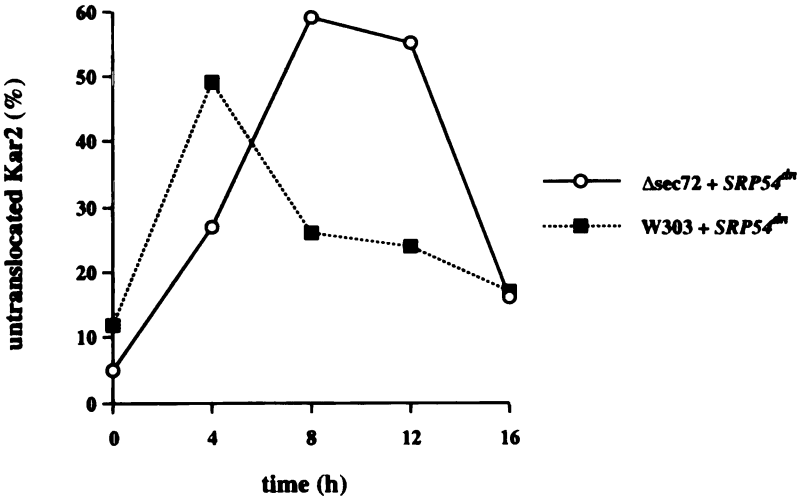
Unknown

Unknown*

Figure 3-3b

Figure 3-4: Several ER membrane proteins are tested for their ability to adapt to the loss of the SRP pathway. Wild type strains or deletion strains for *SEC71* (top panel), *SEC72* (middle panel), and *SSH1* (bottom panel) were transformed as indicated with plasmids containing either the galactose driven $SRP54^{wt}$ or $SRP54^{dn}$ constructs described in Chapter 2. Cells were grown to mid-log phase in raffinose-containing selective synthetic medium, then shifted to the comparable galactose-containing media. Cells were labeled with [^{35}S]-methionine for 7 min, and harvested at the indicated times. Lysates at each time point were immunoprecipitated with anti-Kar2 and analyzed by SDS-PAGE followed by autoradiography. The amount of precursor protein relative to lumenal protein at each time point was quantified and graphed.


untranslocated Kar2 (%)
60
50
40
30
20
10
0
4
8
12
16
time (h)
W303 + SRP54dn
Δsec71 + SRP54wt
Δsec71 + SRP54dn


untranslocated Kar2 (%)
60
50
40
30
20
10
0
4
8
12
16
time (h)
Δsec72 + SRP54dn
W303 + SRP54dn

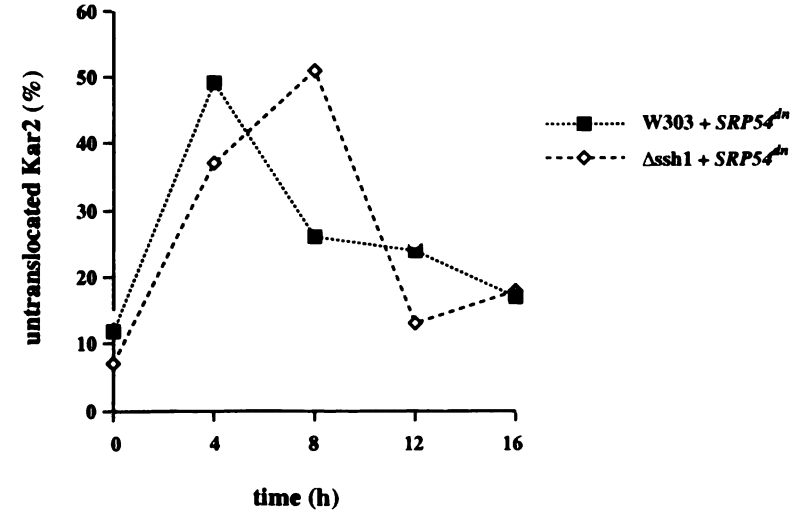
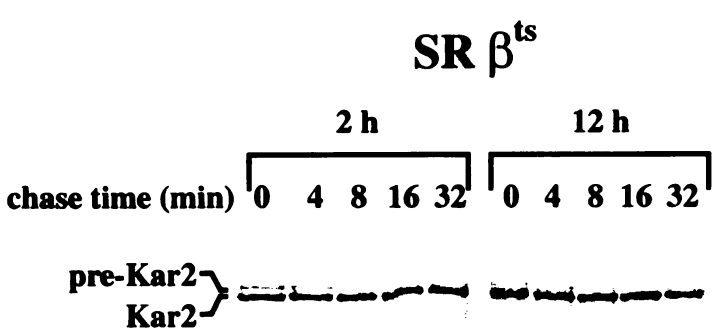
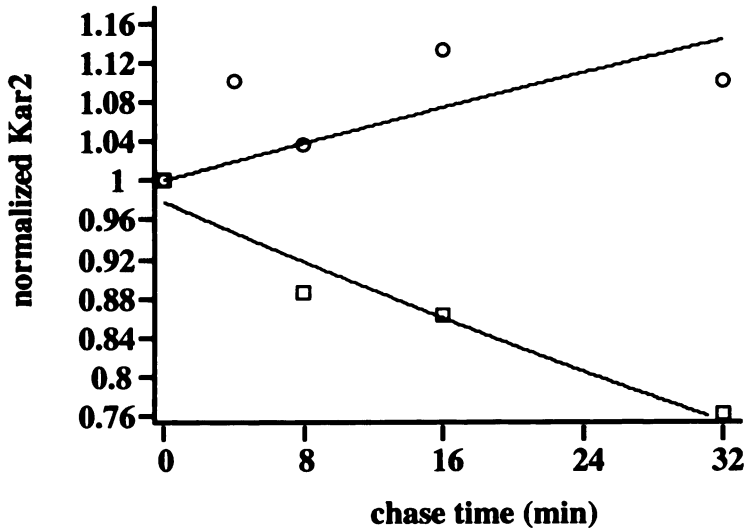

untranslocated Kar2 (%)
60
50
40
30
20
10
0
4
8
12
16
time (h)
W303 + SRP54dn
Δssh1 + SRP54dn

Figure 3-4

Figure 3-5: Stability of untranslocated proteins at early and late time points after SRP pathway disruption using either the SRβ^{ts} mutant (A) or the *SRP54^{dn}* inducible system (B). *srp102(K51I)* cells were grown and labeled at 37°C and *SRP54^{dn}* cells were grown and labeled at 30°C. At 30 min prior to the indicated time point, cells were harvested and washed two times in prewarmed media lacking methionine. Cells were resuspended at $3OD_{600}$U/ml and grown for the remainder of the time point. 20 μCi/OD ProMix (Amersham) was added for 10 min and chased by addition of 2 mM each methionine and cysteine. For the SRβ^{ts} cells, 200 μM cycloheximide was also added. At 0, 4, 8, 16, and 32 min, 1.5 ml aliquots were removed to chilled tubes and quick-frozen in liquid nitrogen. Extracts and immunoprecipitations were performed as described in Chapter 2. Upper panels display the amounts of pre-Kar2 and mature Kar2 recovered at each chase point. Quantitation was performed with a Molecular Dynamics (Sunnyvale, CA) Storm 840 imager and ImageQuant Software, and amount of mature Kar2 (circles) and pre-Kar2 (squares) was plotted versus the chase time. The total amount recovered was normalized to the zero chase time for ease of graphing and visual interpretation.



2 h



12 h

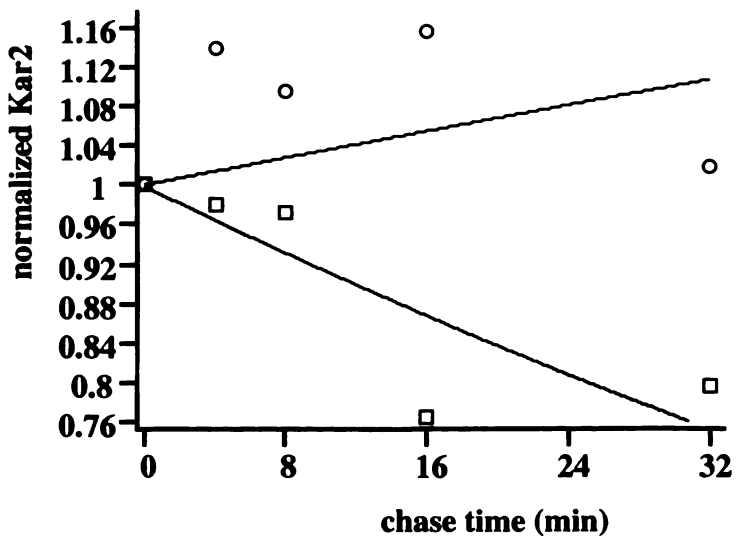


Figure 3-5a

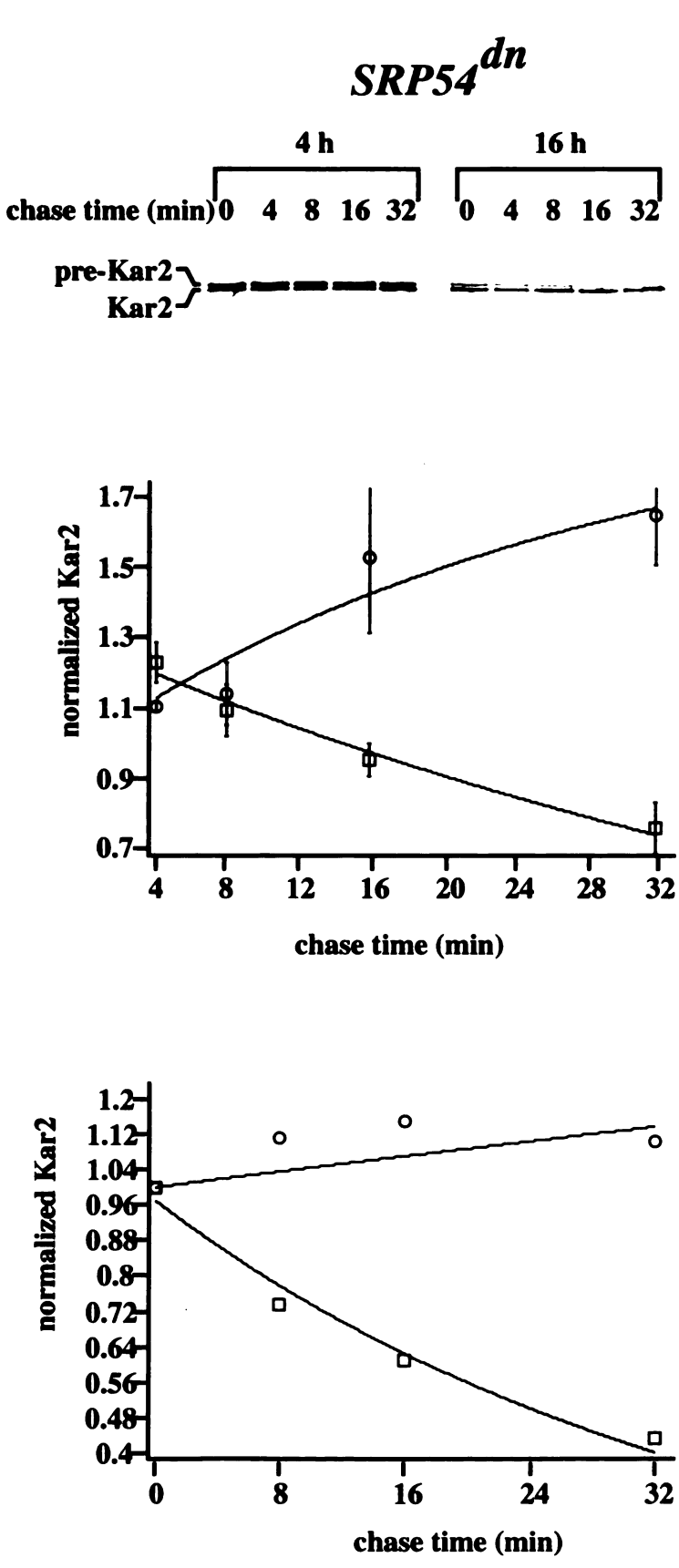
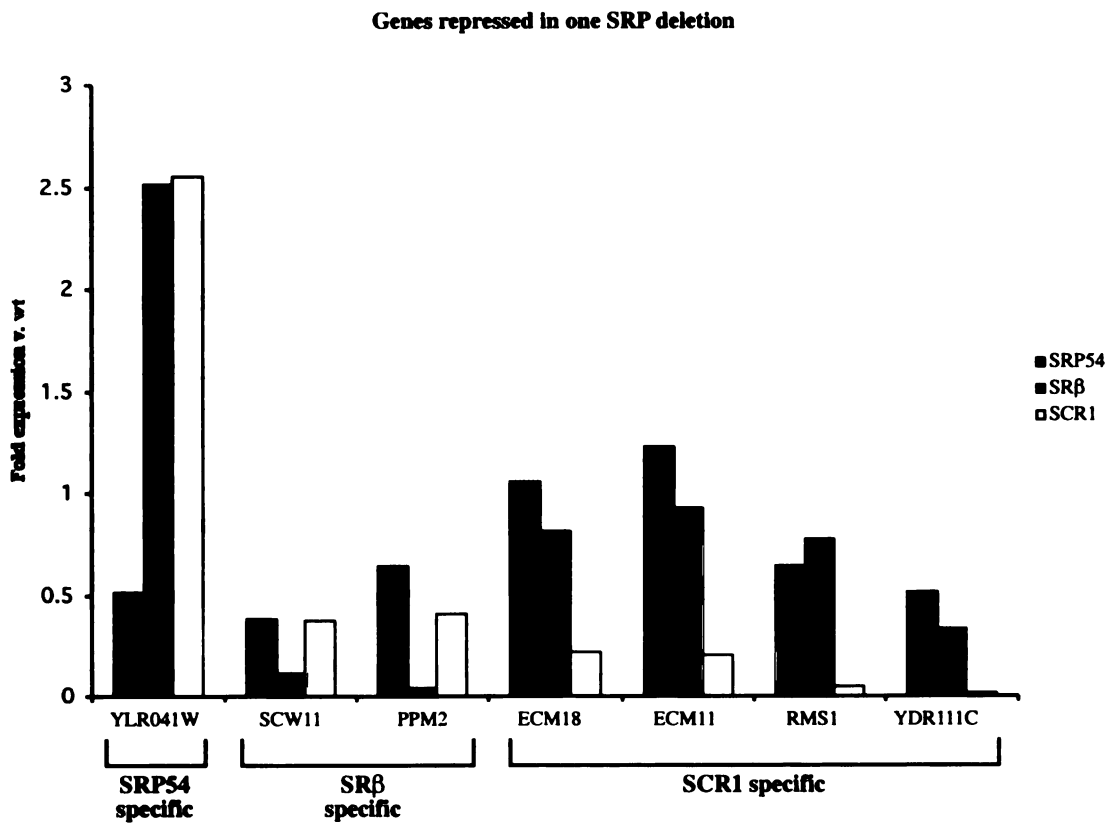

SRP54dn
4 h
16 h
chase time (min) 0 4 8 16 32 0 4 8 16 32
pre-Kar2
Kar2
4 h
normalized Kar2
1.7
1.5
1.3
1.1
0.9
0.7
4 8 12 16 20 24 28 32
chase time (min)
16 h
normalized Kar2
1.2
1.12
1.04
0.96
0.88
0.8
0.72
0.64
0.56
0.48
0.4
0 8 16 24 32
chase time (min)

Figure 3-5b

Figure 3-6: Outliers were identified from Figure 3-1 , and expression ratios for each SRP deletion were plotted. ORFs were grouped according to specificity for each SRP deletion and repression (top panel) or induction (bottom panel).


Genes repressed in one SRP deletion
Fold expression v. wt
3
2.5
2
1.5
1
0.5
0
SRP54
SRβ
SCR1
YLR041W
SCW11
PPM2
ECM18
ECM11
RMS1
YDR111C
SRP54 specific
SRβ specific
SCR1 specific

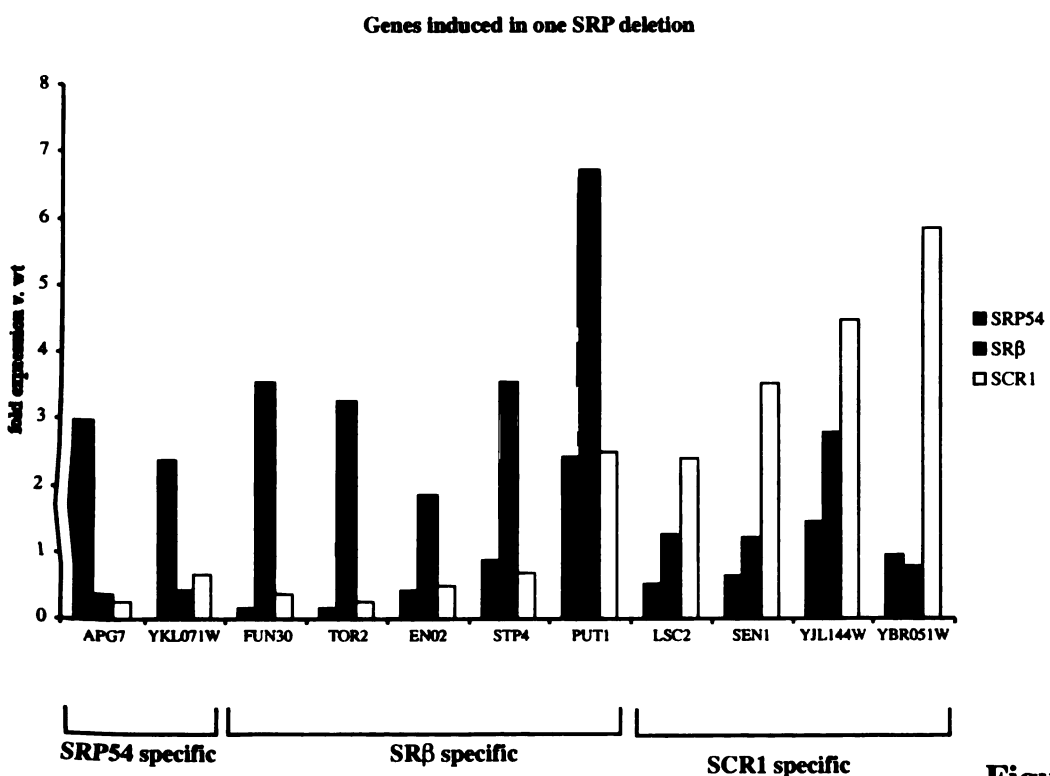

Genes induced in one SRP deletion
fold expression v. wt
8
7
6
5
4
3
2
1
0
SRP54
SRβ
SCR1
APG7
YKL071W
FUN30
TOR2
EN02
STP4
PUT1
LSC2
SEN1
YJL144W
YBR051W
SRP54 specific
SRβ specific
SCR1 specific

Figure 3-6

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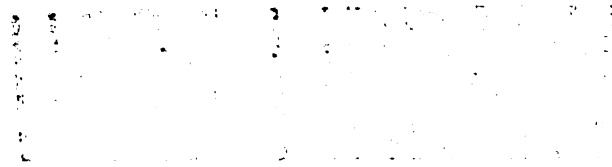

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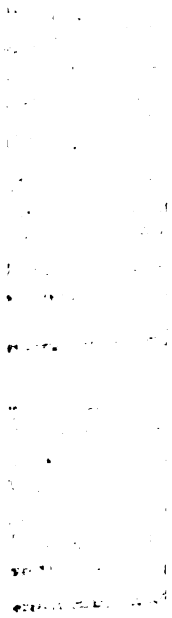
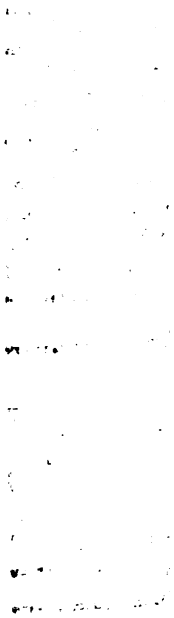
CHAPTER IV:

Summary and Perspectives



This thesis has dealt with the question of how cells survive in the absence of the signal recognition particle (SRP) dependent protein targeting pathway. In Chapter Two, I have described the various approaches we used to address this issue. In the beginning of this project, we focused on the role of the heat shock response, as well as changes at the level of the endoplasmic reticulum (ER). Although it was clear that an induction of heat shock proteins occurred upon loss of the SRP pathway, no individual chaperone deletion had an effect on the cell's ability to adapt. Furthermore, deletions and mutations in proteins involved in translocation at the ER membrane also had no effect on adaptation. It eventually became clear that adaptation to the loss of the SRP pathway is a complex physiological response with multiple levels and that we were lacking adequate tools to dissect the response.

In the middle of this work, sequencing of the entire genome of *Saccharomyces cerevisiae* was completed. This quickly led to the development of high density DNA microarray technologies for expression monitoring. With this tool, we now had the capability of measuring global expression changes throughout the time course of adaptation. This technique allowed us to more fully understand the magnitude of changes occurring upon the loss of the SRP pathway. This also clarified why searching for single genes required for adaptation was a fundamentally flawed approach. Results from global expression monitoring demonstrated that the major transcriptional programs observed upon the loss of the SRP pathway include the induction of chaperone gene expression and the repression of ribosomal genes resulting in a decrease in total protein synthesis. The results suggest that chaperone gene induction serves to protect cells from mislocalized precursor proteins in the cytosol, whereas reduced protein synthesis helps to rescue efficiency in protein sorting by reducing



the load on the protein translocation apparatus. Therefore, we suggest that cells trade speed in cell growth for fidelity in protein sorting to adjust to life without SRP.

Clearly cell survival in the absence of the SRP pathway is a complicated process, and the data we have acquired from expression monitoring has unmasked at least as many questions as it has answered. One question at the forefront is whether protein translocation in the absence of SRP is co- or post-translational. As we have discussed in Chapter Two, many proteins, depending on their signal sequence, show some degree of flexibility in their choice of protein translocation pathway. It seems plausible that, at least for some substrates, protein translocation in the absence of SRP is accommodated post-translationally. However, if the decrease in protein synthesis observed upon the loss of the SRP pathway reflects an overall slowing of elongation, it remains possible that some proteins may be translocated co-translationally even in the absence of the SRP targeting pathway. Experiments to determine whether reduced initiation or slowed elongation is responsible for the decrease in total protein synthesis will help to answer this question. Polysome gradients as well as polysome gradients combined with floatation gradient analysis will be useful in addressing these questions.

Another issue which has not been adequately explored is the role of protein degradation in survival to the loss of the SRP pathway. The question remains as to whether the apparent improvement in translocation efficiency observed in adapted cells is in any part due to increased protein degradation. Although it appears that, for pre-Kar2 in the SRP54^{dn} system, there may be increased protein degradation in adapted cells, many controls to the experiments are still lacking. Because there are two forms of the protein, pre-Kar2 and mature Kar2, techniques must be developed to determine the stability of both forms during

the adaptation time course. Furthermore, if protein degradation is indeed increased, it would be interesting to determine whether this is specific for accumulated precursor proteins or a more generalized degradation pathway.

APPENDIX A:

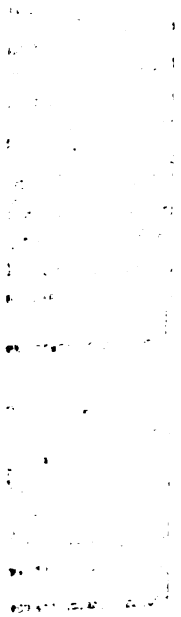
Selection strategies for mutants in sorting to the intermembrane space of the mitochondrion

ABSTRACT

We devised a selection strategy in the budding yeast, *Saccharomyces cerevisiae*, designed to isolate mutations that fail to properly sort proteins to the intermembrane space (IMS) of the mitochondrion. Using the presequence of the IMS-directed cytochrome c_1 protein, we directed various reporter proteins to the IMS and selected for missorting to the matrix by a positive growth phenotype. The most successful of these reporters was Ssc1p, the mitochondrial matrix form of the chaperone Hsp70p (mHsp70p). Using an IMS-directed form of mHsp70p, we isolated 38 mutants apparently defective in sorting to this compartment. All mutants are dominant; no recessive mutants were isolated. Sporulation and/or germination in these mutants were extremely inefficient, and sufficient backcrossing for tetrad analysis was never possible. Cloning by genomic library construction failed to yield results for one mutant tested.

INTRODUCTION

The mitochondrion is believed to have evolved when the precursors of the modern eukaryotic cell engulfed or were invaded by prokaryotic cells. From this, it follows that the organelle is bounded by two membranes which define four subcompartments: the inner membrane (IM), the outer membrane (OM), the matrix, and the intermembrane space (IMS). While mitochondria do contain their own DNA, the vast majority of mitochondrial proteins are encoded in the nucleus. This makes the mitochondrion an excellent organelle to study the targeting, translocation, and sorting of proteins, as proteins must not only be directed to the mitochondrion, but also to one of four subcompartments within the organelle.

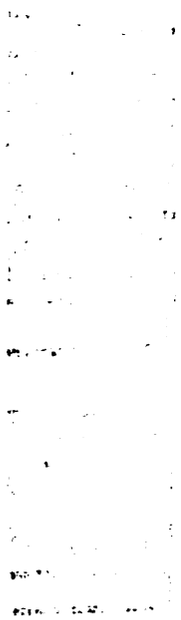


Through a variety of biochemical and genetic approaches, we are now beginning to understand the mechanism and components involved in directing proteins to the matrix of the mitochondrion (for a review see Herrmann and Neupert, 2000), but much less is known about how proteins are sorted at the inner membrane. Furthermore, not all proteins that reside in this compartment arrive by the same mechanism (for a review see Stuart and Neupert, 1996). In some cases proteins are synthesized without any form of cleavable presequence. These proteins do not seem to cross the inner membrane at any point in their targeting. Examples include cytochrome c, which is synthesized as a mature protein that directly inserts into and crosses the outer membrane independent of identified translocation machinery, and cytochrome c heme lyase, which depends on an outer membrane channel used by matrix-targeted precursors.

The IMS proteins we are interested in are synthesized on cytosolic ribosomes with amino-terminal presequences. We have chosen to study the sorting of cytochrome c_1-like proteins because their sorting signals have the general features of secretory proteins destined for the ER as well as export signals in prokaryotes (Hartl et al., 1987; Jensen et al., 1992; von Heijne et al., 1989). Both cytochrome c_1 and cytochrome b_2 contain presequences which are processed twice during their import into mitochondria (Figure A1). The amino-terminal part of these signals targets the protein to the mitochondria and resembles a matrix-targeting signal (Sadler et al., 1984). By itself, this is sufficient for import into the matrix (van Loon et al., 1986). This part of the signal is a positively charged amphipathic α-helix. During import, the precursor is translocated across the inner membrane at least far enough for this part of the signal to be removed by a matrix metalloprotease (Gasser et al., 1982; Ohashi et al., 1982).

The second half of the signal is responsible for sorting the protein to the intermembrane space. It includes a long hydrophobic stretch flanked by both basic and acidic residues (Sadler et al., 1984). Mutational analysis shows that all three of these characteristics are important for the recognition of the signal by the sorting machinery (Jensen et al., 1992). Its removal is catalyzed by the inner membrane protease (IMP) complex in the IMS (Nunnari et al., 1993; Pratje and Guiard, 1986; Schneider et al., 1991) These presequences are necessary and sufficient for the targeting of proteins to the IMS, as they can be attached to many heterologous proteins causing their efficient sorting to this compartment.

A long standing controversy existed as to the possible import and sorting pathways of cytochrome b_2 and cytochrome c_1. One group hypothesized that the hydrophobic stretches of the presequence functioned to "stop-transfer" across the inner membrane (van Loon et al., 1986), while another group hypothesized that the presequence functioned to signal re-export from the matrix into the intermembrane space ("conservative sorting") (Hartl et al., 1987). We chose to use an unbiased genetic approach designed to isolate mutants defective in sorting cytochrome c_1 to the IMS regardless of pathway. Here, I report the results of several attempts to isolate and characterize mutants that are defective in their ability to sort reporter proteins fused to cytochrome c_1 presequences. Using IMS-directed citrate synthase, *URA3*, or matrix directed cytochrome c_1, the selections could not be performed due to various problems with the strains or fusion constructs. Using IMS-directed mHsp70p, the selection generated 38 dominant mutants potentially defective for sorting to the IMS. Attempts to manipulate and clone these strains were not successful.

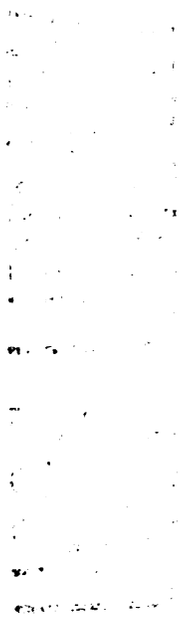


MATERIALS AND METHODS

PCR and Cloning of Reporter Constructs

Mutant presequences generated by site-directed mutagenesis and citrate synthase fusions to cytochrome c_1 presequences were made by Jodi Nunnari, now at the Section of Molecular and Cellular Biology, University of California-Davis. Subcloning was performed into Bluescript based CEN/ARS and integrating vectors (Sikorski and Heiter, 1989) by digestion with *Xho*I and *Eag*I or *Xba*I. All cytochrome c_1 presequence-reporter gene fusions except SSC1 were generated by ligating *Xho*I-*Bam H*I digested presequences to *Bam H*I-*Sac*II fragments. PCR with oligos C1-3 and C1-5 generated IMS-directing presequences and oligos C1-3 and C1-6 generated matrix-directing presequences. Cytochrome c_1 constructs used a PCR fragment generated by oligos C1-1 and C1-2, and *URA3* constructs used a PCR fragment generated by oligos URA1 and URA2B. All oligonucleotides are described in Table 1.

Plasmids containing SSC1 driven by its own promoter and SSC1 driven by the GAL1 promoter were kindly provided by Elizabeth Craig, Department of Biomolecular Chemistry, University of Wisconsin-Madison. Subcloning of the GAL1-*SSC1* fragment to other vectors was performed by digestion with *Eco R*I-*Eag*I. To create the non-regulated, IMS-directed *SSC1* reporter construct, the *SSC1* fragment was generated by PCR with oligos SSC1-1 and SSC1-2 and digestion with *Bam H*I and *Sac*II. The IMS sorting information alone (without the promoter region) was generated by PCR with oligos C1-4 and C1-5 and digestion with *Bam H*I. These two fragments were ligated into the *Bam H*I-*Sac*II digested yeast vector pF201 which contains the glyceraldehyde-3-phosphate dehydrogenase promoter. Further



subcloning to other vectors was done by digestion with *Xho*I and *Sac*II. Relevant plasmids are identified in Table 3.

Construction of Knockout Strains

The citrate synthase knockout was constructed by Jodi Nunnari. This construct was transformed into yeast strain JKR101. The cytochrome c_1 knockout was made with a full length cytochrome c_1 containing vector digested with *Age*I and *Spe*I. The ends were blunted with T4 polymerase and ligated to a blunt *Sph*I *LEU2A* fragment. The construct was excised by digestion with *Xho*I and *Xba*I and introduced into a *leu2A* diploid strain (W303) by one step gene replacement (Orr-Weaver et al., 1981) using the PEG-LiOAc procedure (Elble, 1992). Integration into the *CYT1* locus was confirmed by Southern blotting (Maniatis et al., 1982).

The *SSC1* gene was disrupted by one step gene replacement (Orr-Weaver et al., 1981). A 146nt 5' fragment was produced by PCR using oligos KO1 and KO2 followed by digestion with *Sma*I and *Xho*I, and a 186nt 3' fragment was generated with oligos KO3 and KO4 by PCR and digestion with *Sma*I and *Sac*II. These fragments were ligated into the yeast integrating vector pRS305 digested with *Xho*I and *Sac*II. The resulting plasmid was digested by *Sma*I and transformed into a diploid yeast strain using the PEG-LiOAc procedure. Integration into the *SSC1* locus was confirmed by Southern blotting (Maniatis et al., 1982).

Mutagenesis

Strains for all selections were mutagenized with UV irradiation to 35-70% kill as described (Guthrie and Fink, 1991).

Construction of Genomic Libraries

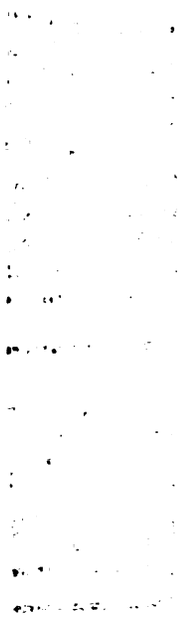
Genomic libraries were constructed as described (Sambrook et al., 1989). Genomic DNA from mutant yeast strains was partially digested on ice with *Sau*3A. 5-12 Kb fragments were gel purified and ligated into dephosphorylated, *Bam*HI digested pRS316. Restriction digests demonstrated at least 50% of the library contained 5-12 Kb inserts, and we conservatively calculated 4000 transformants to represent coverage of the genome one time. The library was checked for representation by PCR of 3 genes: *CYB2, LEU2,* and *HIS3.*

RESULTS

Selections on non-fermentable carbon sources: citrate synthase

To identify components of the mitochondrial IMS protein sorting machinery of *Saccharomyces cerevisiae*, we used a positive selection strategy for mutants with defects in sorting. This genetic approach is a modification of one that has been used successfully to isolate mutants in the yeast secretory pathway (Deshaies and Schekman, 1987) and in the mitochondrial protein import machinery (Maarse et al., 1992). The approach is based on the mislocalization of a fusion protein that is efficiently sorted to the IMS but that has activity only in the matrix (Figure A2).

The first attempt at this selection used citrate synthase (*CIT1*) as a reporter. Citrate synthase is the enzyme that catalyzes the condensation of oxaloacetate and acetyl-CoA to form citrate in the matrix of the mitochondrion. This function is required for respiration, and consequently SMY100, a yeast deletion of *CIT1* (see Table 2 for strains), cannot grow on non-fermentable carbon sources like acetate. This strain can be complemented by a plasmid

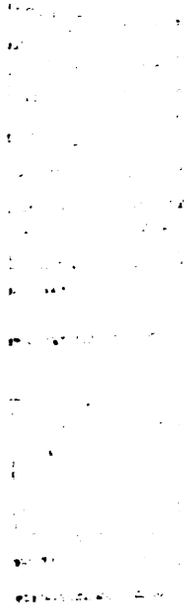


encoding a matrix-directed citrate synthase fusion protein. When SMY100 carries a plasmid encoding an IMS directed fusion protein, the strain shows a significant reduction in growth rate but is not inviable.

Nevertheless, the existing minimal level of complementation prevents this selection from working. When mutagenized and plated at high cell densities used in the selection, mutants can scarcely be distinguished from the lawn of background growth. Furthermore, every mutant that was isolated for characterization proved to be plasmid linked. Presumably, this is because even weak mutations in the sorting signal would be adequate to allow missorting and growth given that the fusion protein is already inefficiently targeting. So, the high level of background growth together with a cumbersome secondary screen for plasmid linked mutants made this reporter system undesirable.

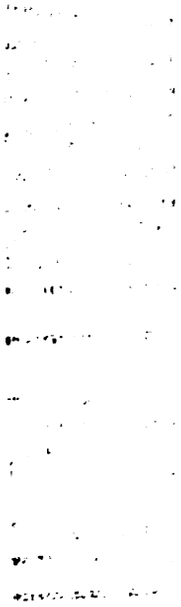
Selection on non-fermentable carbon sources: cytochrome c_1

At the same time, I used the full-length cytochrome c_1 (*CYT1*) for a similar selection strategy (Figure A3). This protein is a component of the oxidative phosphorylation machinery, so it is required for respiration and therefore growth on the non-fermentable carbon sources ethanol and glycerol. Cytochrome c_1 can be missorted to the matrix (where it is not functional) by a variety of different signal sequence mutants (Jensen et al., 1992). Several different mutant signal sequences were created by site directed mutagenesis (pSM40, pSM16, pSM42, pSM43, see Table 3). The single point mutation A48P, while clearly missorting, proved to be very unstable and provided the cytochrome c_1 deletion, SMY103, varying degrees of growth on ethanol and glycerol. Therefore, we created a different point mutation (L52P), combined the two point mutations (SMY106), and created deletions of the



hydrophobic core of the sorting information (Δaa157-179, SMY105). All of these signal sequence mutations appeared stable and afforded SMY103 no complementation on ethanol and glycerol. SMY105 and SMY106 were used in subsequent experiments.

In the course of making these strains, it was determined that the mitochondrial genome of SMY103 was extremely unstable. After growth on dextrose for 2 days, it was found that approximately 17% of independent colonies tested (n=18) had lost all or part of their mitochondrial genome (rho^-) as judged by mating to a rho^0 strain and checking for growth on non-fermentable carbon sources. Because this is precisely the phenotype expected for the strains to be used in the selection, it was critical to find a way to stabilize the mitochondrial genome. To that end, I determined the rho^- frequency of SMY103 when maintained on galactose. Although galactose is a fermentable carbon source, transporters required for its utilization depend on the electrochemical proton gradient of the mitochondrion (Lagunas, 1993), and it is possible that this requirement could help prevent the loss of mitochondrial DNA. Indeed, when SMY103 was grown on galactose for several generations (1-2 days), 100% of independent colonies tested (n=24) remained rho^+. Because of this finding, SMY103 and all strains derived from it were maintained on galactose. The selection for mitochondrial function proved to be too good, however, as the strains carrying the point mutated signal sequences now displayed very heterogeneous growth where there was none previously. Furthermore, even constructs with deletions in the signal sequence appeared to be unstable or easily suppressed. For example, after transformation of SMY105 with a high copy yeast genomic DNA library, approximately 80% of transformants had a positive growth phenotype, but none of the transformants tested was dependent on the library plasmid for this growth. Because of the overwhelming number of false positives in this

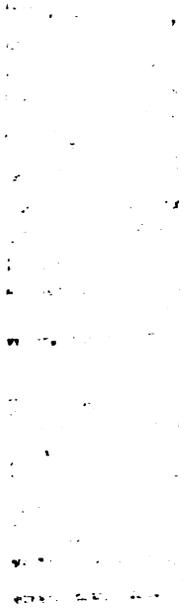


selection strategy as well as difficulties with strain maintenance, this approach was not desirable.

Selection on a fermentable carbon source: *SSC1*

The type of mutation we seek may occur only rarely because the selective media in the previous two strategies is a non-fermentable carbon sources, on which oxidative phosphorylation is the sole source of ATP for yeast. Consequently, a balance must be reached that allows the reporter to be missorted to its functional location in the IMS or matrix without significantly altering the levels of remaining components of the oxidative phosphorylation machinery. Hence, we designed a selection strategy that uses a reporter, *SSC1*, which is not involved in respiration (Figure A4). A major benefit this approach is the ability to select on glucose. On fermentable carbon sources such as this, the yeast cell derives its ATP from glycolysis, and the major function of the IMS, oxidative phosphorylation, is bypassed. Because of this and combined with the results from our citrate synthase and cytochrome c_1 selections, we felt this strategy was the most likely to succeed.

SSC1 is an essential gene involved in protein import and folding in the matrix of the mitochondrion. I constructed a complete knockout of this gene in the diploid strain W303 in the presence of a plasmid (pEC557, E. Craig) containing *SSC1* driven by the regulated promoter GAL1 (SMY110). This strain was viable on galactose, but quickly died when switched to glucose. Growth of SMY110 on glucose was restored by plasmids containing *SSC1* fused to a defective IMS (A48P, L52P) sorting signal but not the wild type signal (pSM61 to create SMY113).

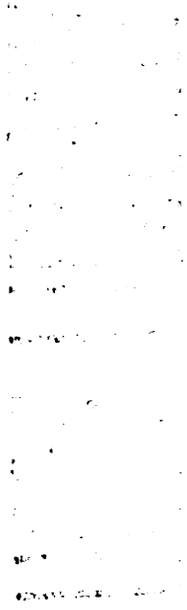


SMY113 was subsequently used for mutagenesis and selection for growth on glucose containing medium. 110 mutants were isolated and carried through secondary screens against cis mutants, galactose derepressed mutants, and reporter independent mutants (see Table 4). The results were somewhat surprising. The percentage of mutants which were plasmid linked (presumably signal sequence mutations) was very high (~60%). Furthermore, very few galactose derepression mutants were isolated (~2%). Finally, the 38 mutants that survived these screening requirements were all dominant. No recessive mutations were isolated although it is conceptually realistic to expect that they would be found.

However, given that the mutants were all unlinked to the reporter plasmid yet dependent on its presence, these mutants were strong candidates for components of the sorting machinery. Furthermore, the dominant mutants could be the result of the high degree of overexpression of the reporter protein, such that even in the presence of the wild type sorting machinery, enough of the reporter can be missorted to the matrix (see below).

Characterization of mutants

In order to further characterize these mutants, I next attempted to backcross these mutants to the parent strain (SMY111). Many mutants failed to sporulate, while others failed to germinate on either galactose/sucrose or on glucose. Ultimately, 16 of the 38 mutants could be backcrossed one time. 7 of these 16 could be backcrossed a second time, and, in most cases, backcrossing did not alleviate the sporulation and germination problems. One mutant, SMY196, was most easily backcrossed (although it never germinated well enough to allow tetrad analysis of the mutant phenotype), and this mutant was chosen for further analysis and cloning.



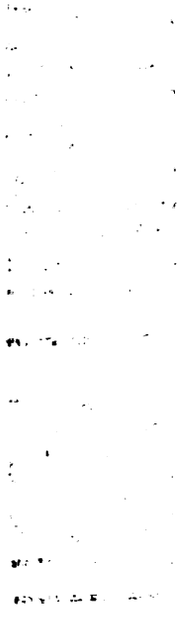
If the dominant mutants were truly defective in sorting to the IMS, then it would be expected that intermediate forms of the IMS-directed *SSC1* reporter would accumulate. In all mutants tested, there was an accumulation of a slower migrating form of Ssc1p when compared to the wild type. Based on molecular weight, this is presumably the intermediate form. However, this experiment was not adequately controlled. Attempts to generate a standard for the intermediate form failed; in two different intermembrane space protease deficient strains (an inactive mutant and a complete null allele) in which intermediate forms of such IMS proteins should accumulate no protein was ever observed by Western blotting. An additional problem is that, given the seemingly high susceptibility of this signal sequence to mutations, I can not rule out that the intermediate accumulation observed was not partially due to the accumulation of random signal sequence mutations. However, when this experiment was repeated for SMY196 (Figure A5) with a newly transformed, uncompromised IMS- directed *SSC1* reporter plasmid, the slower migrating form of the protein (presumably intermediate) was again observed although to a lesser degree. Therefore, despite the limitations discussed, it appears that the mutants are accumulating the IMS-directed fusion protein in a different manner than the wild type strain that is consistent with the expected behavior of a sorting mutant.

I attempted to clone SMY196 by two different methods. First, we observed that the diploid form of this mutant accumulated less intermediate than the haploid form (Figure A5). We therefore wondered if over-expression of the wild type form of the mutant gene could revert the mutant's positive growth phenotype. To test this, I transformed SMY196 with a *S. cerevisiae* overexpressed GAL-cDNA library (Liu et al., 1992), but no cDNAs were found to cause reversion of the mutant phenotype. Next, following the classical method for cloning a

dominant mutant, I constructed a genomic library from SMY196. Representation of several different genes was confirmed by PCR, and the library was transformed into a wildtype strain. Transformants representing approximately seven genomes were screened, and approximately 8% of these had positive growth phenotypes. Positives were screened by colony hybridization and PCR to eliminate those containing *SSC1*. 46 transformants were then retransformed into the original strain to confirm the growth phenotype, but no plasmids could be rescued upon retransformation.

DISCUSSION

It remains possible that the dominant mutants could be cloned with new techniques that have been developed since the time this project was closed such as cloning by transposon insertion (C. Patil, unpublished technique and (Ross-Macdonald et al., 1997)). However, most success in this field has come through the use of biochemical techniques such as crosslinking and *in vitro* assays. Success with genetic approaches has been limited mostly to the study and isolation of mutant targeting sequences (Beasley et al., 1993; Jensen et al., 1992). Since this project was ended, a clearer picture of mitochondrial import into the matrix has emerged. In the outer membrane, the TOM (translocase of the outer membrane) complex has been purified and characterized. This complex consists of 2 receptors, Tom20 and Tom70, as well as a general insertion pore formed by Tom 40, Tom22, Tom7, and Tom6. Once translocated proteins pass through the TOM channel, they interact with the TIM complex (translocase of the inner membrane). This complex consists of integral membrane proteins Tim17 and Tim 23, as well as matrix protein Tim44 in a 2:2:2 ratio. Energy for translocation across this complex comes from the membrane potential as well as from



preprotein binding by matrix-Hsp70 which associates with Tim44 in an ATP-dependent manner (for a review see Herrmann and Neupert, 2000).

For proteins destined to the intermembrane space, it is now known that several different pathways exist to accommodate both protein sorting as well as both sides of the long standing feud on the subject. Cytochrome b_2 has been shown to require the TIM23 complex for its sorting to the IMS. Rather than being imported, translocation of these proteins is arrested at the TIM23 complex (a "stop-transfer" model), followed by lateral insertion into the lipid bilayer, a process which is not understood (Bomer et al., 1997). Other proteins seem to be completely imported into the matrix followed by sorting back out to the IMS (a "conservative sorting" model). The only protein identified to play a role in this sorting is Oxa1. This protein is required for sorting but does not function in translocation, and the mechanism of transport is still unclear (He and Fox, 1997; Hell et al., 1997). Finally, a third pathway to the inner membrane was also discovered recently which is used mainly by polytoopic membrane proteins with internal targeting sequences. These proteins do not use the TIM23 complex, but rather are bound in the IMS by a complex made up of Tim9 and Tim10. The preproteins are then transferred to a TIM22 complex consisting of integral membrane proteins Tim22 and Tim54, as well as a peripheral Tim9/Tim10/Tim12 complex. This complex then directly mediates the insertion of the protein into the inner membrane (reviewed in Herrmann and Neupert, 2000).

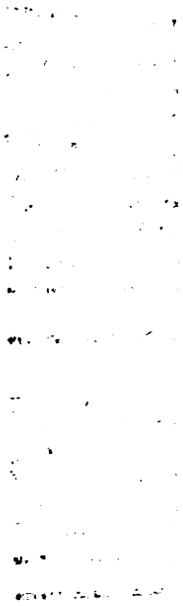


Table 1: Oligos used in this project (location is expressed as nucleotide position relative to starting AUG)

Oligo	Sequence	Direction	Location
URA1	5'CGCGGATCCTCGAAAGCTACATATAAGG3'	sense	+4
URA2B	5'TCCCCGCGGGAAGCTCTAATTTGTGAG3'	antisense	+861
C1-1	5'CGCGGATCCGCTGAACACGGATTGCAC3'	sense	+193
C1-2	5'TCCCCGCGGGCAGCAGTATCTCAGTAC3'	antisense	+1206
C1-3	5'CCGCTCGAGATCTTATTAGTTCACATGG3'	sense	-790
C1-4	5'CGGGATCCCGGAATTCCGATGTTTTCAAATCTATC 3'	sense	+1
C1-5	5'CGGGATCCTGCGGTCATAGCTTCGGCAGT3'	antisense	+200
C1-6	5'CGGGATCCTTTCGAGAGGGTCCTTTGAGCCC3'	antisense	+48
SSC1-1	5'CGCGGATCCCAAGGTTCCGTCATCGG3'	sense	+84
SSC1-2	5'TCCCCGCGGCATCATTACCGTCTGGG3'	antisense	+2422
KO1	5'CGGCCCGGGATCGCAATGGTACAATGTGC3'	sense	-361
KO2	5'CGCCGCTCGAGCACCGCAACCGTAAGCGG3'	antisense	-215
KO3	5'CGGCCCGGGCATCATTACCGTCTGGG3'	antisense	+2422
KO4	5'TCCCCGCGGGATGGGCTTTCACTCCAC3'	sense	+2236

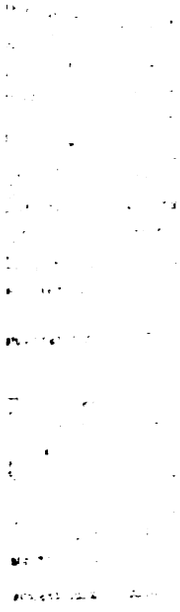


Table 2. Strains used in this study

Strain	Genotype
W303	ade 2-1/ade 2-1; trp1-1/trp1-1; leu2-3,112 /leu2-3,112; his3-11/his 3-11; ura3-1/ura3-1; can 1-100/can1-100; MATa/MATα
JKR101	leu2-3,112 his 4-519 ura3-_ ade2-1 MATα
SMY100	leu2-3,112 his 4-519 ura3-_ ade2-1 cit1::URA3 MATα
SMY101	SMY100[pSM6]
SMY103	ade 2-1trp1-1 leu2-3,112 his 3-11 ura3-1can1-100 cyt1::LEU2MATα
SMY104	SMY103 [pSM38]
SMY105	SMY103 [pSM40]
SMY106	SMY103 [pSM43]
SMY110	ade 2-1trp1-1 leu2-3,112 his 3-11 ura3-1can1-100 ssc1:LEU2MATα [pEC557]
SMY111	ade 2-1trp1-1 leu2-3,112 his 3-11 ura3-1can1-100 ssc1:LEU2MATa [pEC557]
SMY112	ade 2-1trp1-1 leu2-3,112 his 3-11 ura3-1can1-100 ssc1:LEU2MATα [pSM70]
SMY113	SMY110 [pSM61]
SMY114	SMY111 [pSM61]
SMY196	ade 2-1trp1-1 leu2-3,112 his 3-11 ura3-1can1-100 ssc1:LEU2MATα [pSM61], mutant X

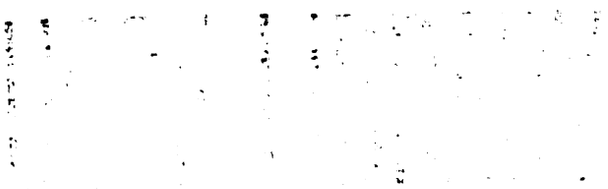


Table 3: Plasmids used in this study

Plasmid	*Identification*
pSM6	IMS-*CIT1* in pRS315
pSM38	IMS-*CYT1* in pRS316
pSM40	IMS(Δaa157-179)-*CYT1* in pRS316
pSM16	IMS(A48P)-*CYT1* in pRS316
pSM42	IMS(L52P)-*CYT1* in pRS316
pSM43	IMS(A48P,L52P)-*CYT1* in pRS316
pEC557	GAL1-*SSC1* in YCp50
pSM70	GAL1-*SSC1* in pRS313
pSM61	IMS-*SSC1* in pF201

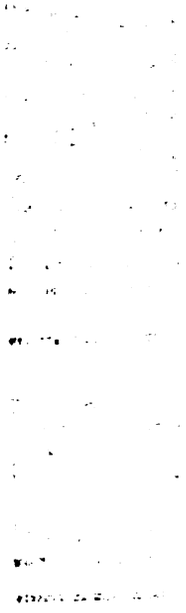


Table 4: Mutants tested from SSC1 screen

Secondary Screen	Number of Mutants Eliminated	Percentage of Mutants
Galactose derepression	1	1 %
Plasmid linked/signal sequence mutations	65	59%
Reporter independent	3	3%
Not determined (too sick to transform)	3	3%
Eliminated	72	66%
Remain to characterize	38	34%
Total	110	100%

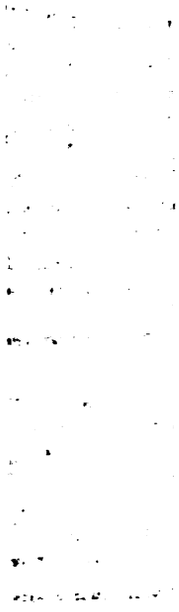
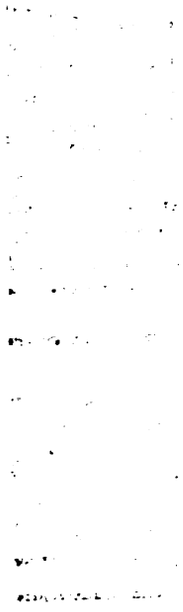
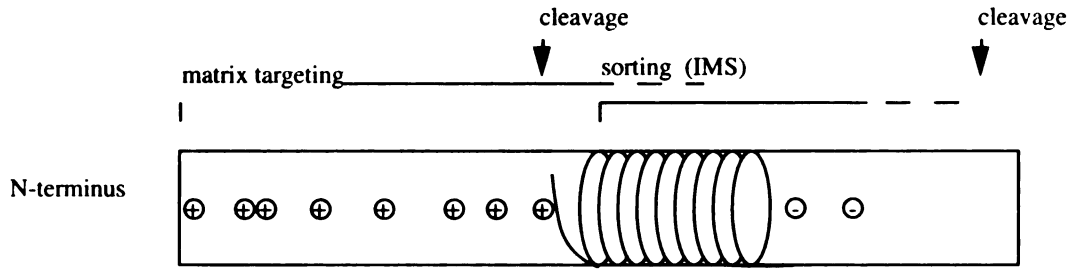


Figure A-1: Signal sequences direct proteins to their proper location within the mitochondrion. The upper panel shows the general features of bipartite intermembrane space sorting signals. The amino-terminal part of the signal directs the protein to be imported into the mitochondria and resembles a matrix-targeting signal. This part of the signal contains residues which form a positively charged amphipathic α-helix, and it is removed upon import across the inner membrane of the mitochondrion by cleavage by a matrix metalloprotease. The second half of the signal is responsible for sorting the protein to the intermembrane space. It includes a long hydrophobic stretch flanked by both basic and acidic residues, and it is removed in the IMS by the inner membrane protease complex. The lower panel shows the sequence of the cytochrome c_1 signal. Charged residues are indicated by blue (+) or (-) symbols, and hydrophobic stretches are indicated by red coloring. Cleavage sites are indicated with arrows.




cleavage
cleavage
matrix targeting
sorting (IMS)
N-terminus

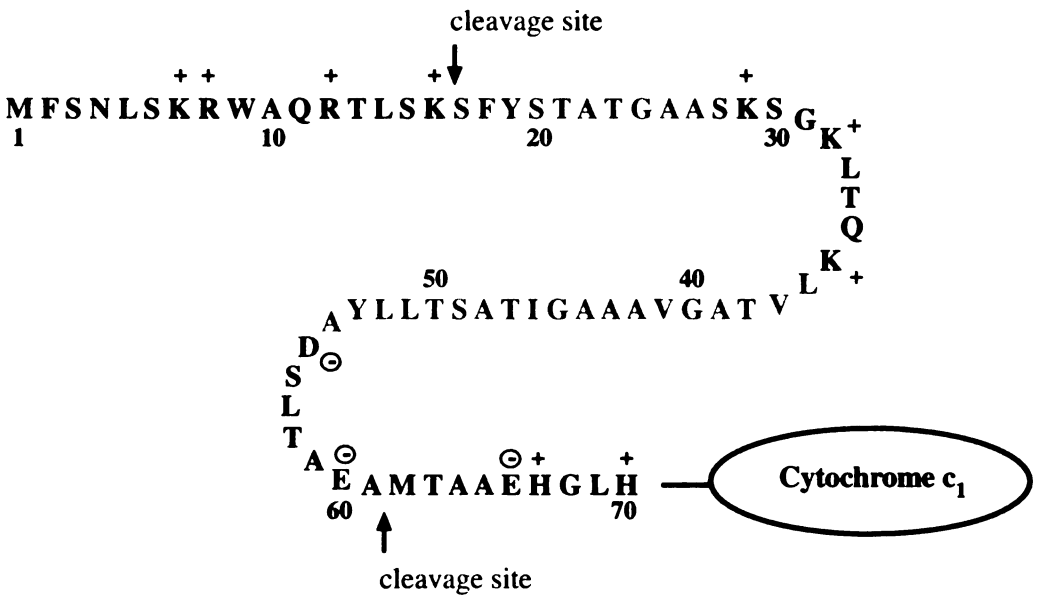
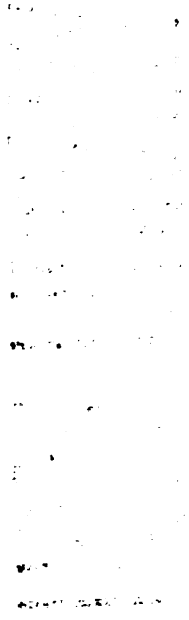
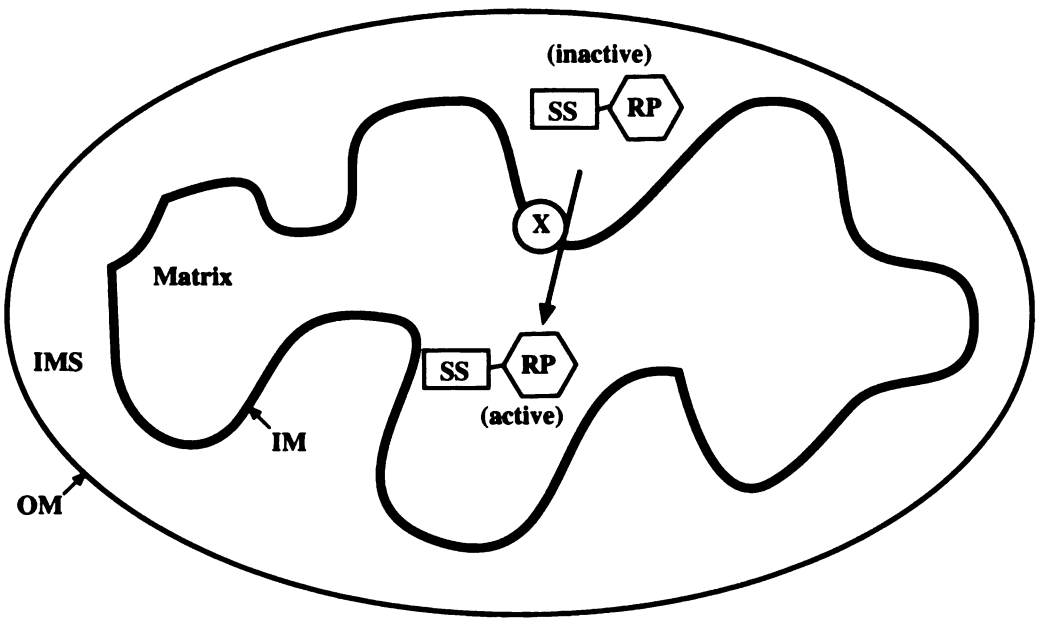

cleavage site
MFSNLSKRWAQRTLSKSFYSTATGAASKSGKLTQKLYLLTSATIGAAAVGATVADSLTAEAMTAAEHGLH
1
10
20
30
40
50
60
70
Cytochrome c1
cleavage site

Figure A-1

Figure A-2: General strategy for selection of intermembrane space (IMS) sorting mutants. A reporter protein (RP) is fused to a signal sequence (SS) which directs the protein to the IMS where it is not functional. Sorting mutants (X) are sought which missort the fusion protein to the matrix where it is active. The activity of the protein provides a selectable phenotype, generally growth on a specific medium.





Strain	Location of RP	Activity of RP
wt	matrix	+
SS-RP	IMS	–
Sorting mutant (X)	matrix	+

Figure A-2

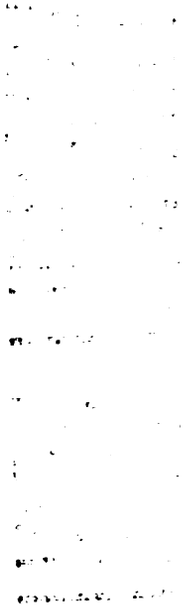
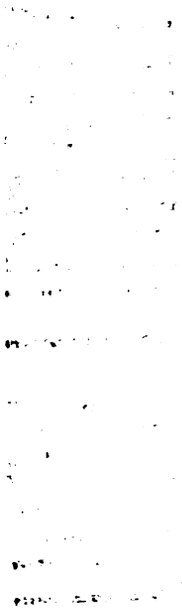
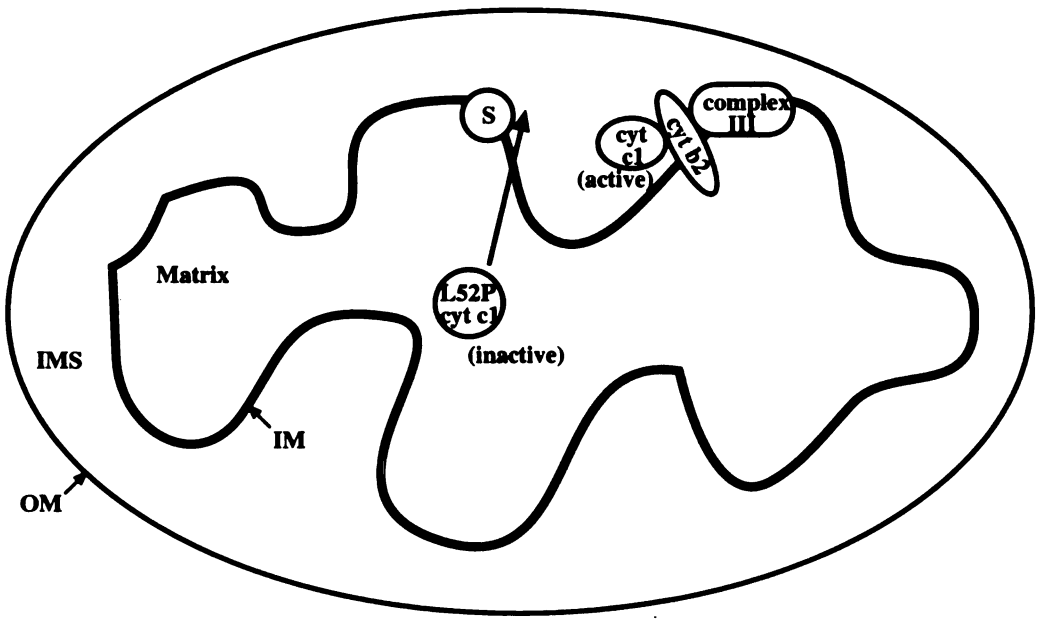


Figure A-3: Selection for suppressors of mutations in the cytochrome c_1 presequence. Cytochrome c_1 is missorted to the matrix of the mitochondrion due to mutations within its presequence. There, it is not functional, and cells can not grow on non-fermentable carbon sources such as ethanol and glycerol. Suppressor mutants (S) are sought which properly sort the mutant cytochrome c_1 to the IMS, allowing growth on ethanol and glycerol.





Strain	Location of Cyt c_1	Growth on Ethanol glycerol
wt	IMS	+
Δcyt c1	deleted	–
L52P Cyt c_1	matrix	–
Suppressor mutant	IMS	+

Figure A-3

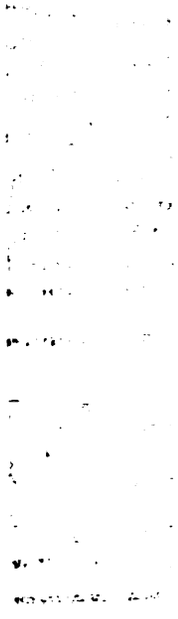
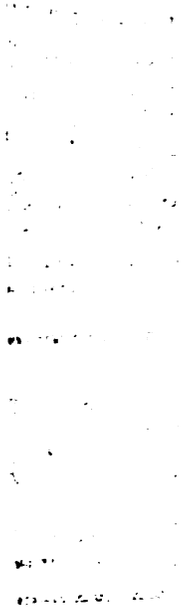
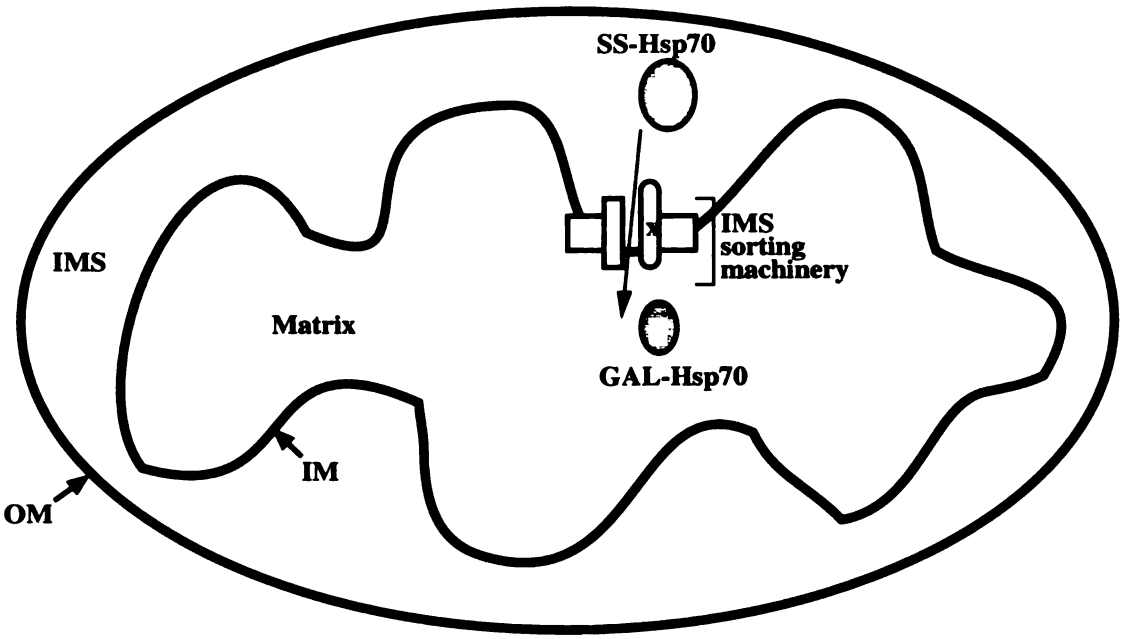


Figure A-4: Selection for sorting mutants using mHsp70p. *SSC1* (encoding mHsp70p) is an essential gene, and strains with genomic deletions must carry the gene in a functional form on a plasmid to survive. The selection is performed in a Δ*ssc1* strain carrying as plasmids both a wildtype *SSC1* gene under the control of the GAL1 promoter (GAL-mHsp70p) and a second copy of *SSC1* fused to the presequence of cytochrome c_1 (SS-mHsp70p). In a normal cell, this strain will grow on galactose-containing media but dies when switched to glucose-containing media. We select for mutants which missort SS-mHsp70p to the matrix by selecting for viable mutants on glucose-containing media.





Strain	Location	GROWTH ON: Galactose	GROWTH ON: Glucose
wild type	matrix	+	+
Δssc1	deleted	-	-
Δssc1 +GAL-Hsp70	matrix	+	-
Δssc1 +SS-Hsp70 +GAL-Hsp70	IMS	+	-
Above strain with sortingmutant (x)	matrix	+	+

Figure A-4

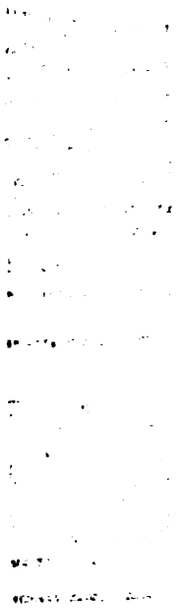
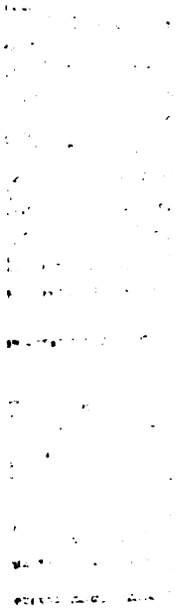


Figure A-5: A dominant mutant accumulates the intermediate form of the IMS-directed *SSC1* reporter. Whole cell extracts were made in a wildtype or a mutant strain as described in Chapter 2 and analyzed by SDS-PAGE and Western blotting for mHsp70p. In both haploid and diploid wildtype strains, only the mature form of mHsp70p was observed. However, in the haploid mutant strain tested, a slower migrating, intermediate form of mHsp70p accumulated nearly exclusively. When the mutation was present in only one copy in a diploid strain, some appropriate sorting occurred as judged by the presence of a small amount of mature-mHsp70p.



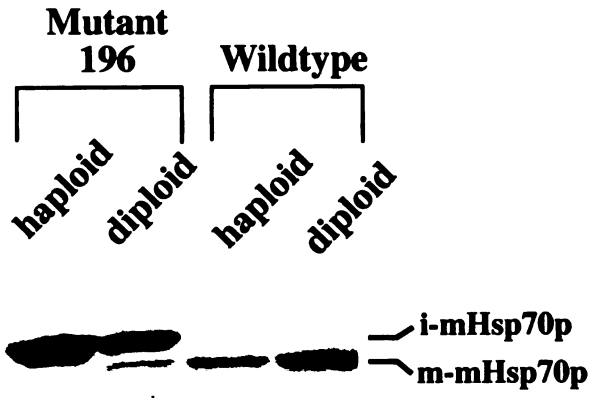
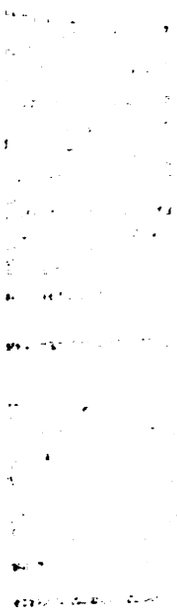

Mutant
196
Wildtype
haploid
diploid
haploid
diploid
i-mHsp70p
m-mHsp70p

Figure A-5



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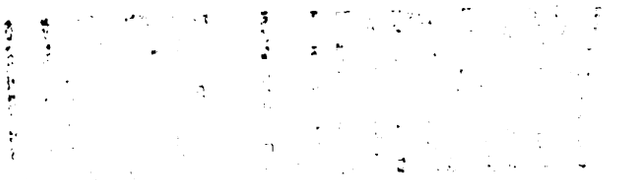

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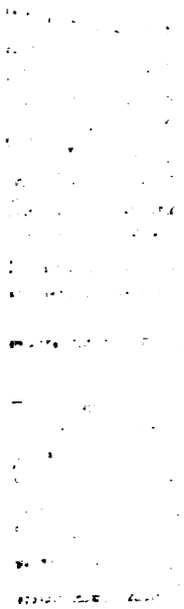

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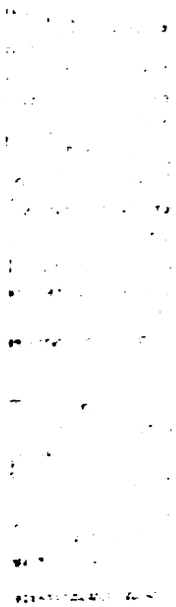

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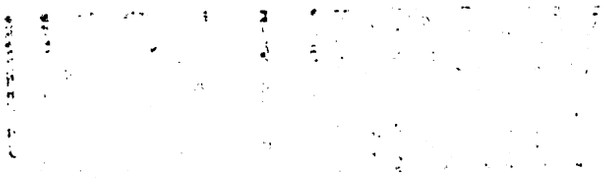

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