Title
Function of the Pho85-Pcl kinase in cell cycle progression

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FUNCTION OF THE PHO85-PCL KINASE IN CELL CYCLE PROGRESSION

by

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THESIS

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Introduction

There are many internal and external signals that feed into the eukaryotic cell cycle and allow for its proper regulation. Molecules involved in signal transduction can inhibit or stimulate the machinery of the cell cycle such as cyclins and cyclin dependent kinases which in turn will inhibit or stimulate the proliferation of certain cells and tissues.

A model organism that has proven useful for studying these types of events is *Saccharomyces cerevisiae*, commonly known as budding or baker's yeast. In *S. cerevisiae*, progression through the cell cycle has been thought to be controlled mainly by a single essential cyclin dependent kinase (CDK), Cdc28. However in recent work (Espinoza et al. 1994 and Measday et al. 1994) it has been shown that a nonessential CDK, Pho85, may also play a role in cell cycle progression. The main question this thesis tries to begin to answer is what role Pho85, together with its associated cyclins, has in cell cycle progression in budding yeast. The way I want to investigate this question is to find new substrates or downstream effectors of the Pho85-cyclin complexes using a genetic approach.

The aspect of substrate specificity given by the cyclin partner is now known but to date scientists have only discovered a handful of substrates of cdk's. One could imagine the potential information gained by understanding the intricacy of cyclin dependent kinases and their many substrates. Discovering substrates of the CDK's coupled with

Background

The eukaryotic cell cycle can be described as a round of DNA replication and subsequent segregation (mitosis) of the DNA into the mother and daughter cell. DNA replication and mitosis are separated by two gap phases, G1 and G2. In budding yeast entry into the cell cycle is marked by a place in late G1 called START (reviewed by Kim Nasmyth, 1993). Passage through START commits the yeast cells to replicate their DNA and segregate the DNA to the mother and daughter cells. A morphological marker which indicates passage through START and commitment to the cell cycle is budding. Cdc28, the master regulatory protein associates with various sets of cyclins throughout the cell cycle. The Cdc28-cyclin kinase activity activates other proteins which in turn activate the transcription of proteins needed at precise times in the cell cycle.

As noted earlier *PHO85* encodes a cyclin dependent kinase with approximately 50% identity to *CDC28*. Pho85, together with its cyclin subunit Pho80, is known to be a negative regulator of *PHO5* transcription (*PHO5* encodes a secreted acid phosphatase). When cells are grown in high phosphate media Pho80-Pho85 phosphorylates the transcription factor Pho4 (Kaffman et al. 1994). Phosphorylation of Pho4 causes its rapid export from the nucleus (O'Neill et al., submitted). However, in media with low phosphate, the activity of the kinase is inhibited by the cyclin dependent kinase inhibitor Pho81. Pho4 is then hypophosphorylated and competent to enter the nucleus and bind to the upstream regulatory regions in the *PHO5* promoter which activates *PHO5* transcription.

Recently there have been two other proteins found to associate with Pho85 in vivo called Pcl1 and Pcl2 (Espinoza et al. 1994, Measday et al. 1994). These Pho85 cyclin like molecules, Pcl1 and Pcl2, as well as other G1 cyclins such as Cln1 and Cln2 contain the the cell cycle box in their upstream regulatory regions known. The Swi4/Swi6 transcription factor complex binds at these sites and causes the increase in transcription of these genes. Interestingly, Cdc28 does not associate with Pcl1 and Pcl2. Kinase activity against Pho4 has been shown to be dependent upon Pho85 and not Cdc28. Also when HA tagged Pcl1 is immunoprecipitated with 12CA5 antibodies, it associates with Pho85 and not Cdc28. The function of Pcl1 and Pcl2 as well as their Pho85 interaction is not known.

There has been, however, compelling genetic evidence that supports the hypothesis that Pho85 together with its Pcl cyclin counterpart does function in the G1-S phase transition. Cdc28 associates with Cln1, Cln2, and Cln3 in G1 and these cyclin-kinase complexes causes progression through START. Yeast are viable as long as they have at least one functional Cln, but cells mutant for all three cyclins are inviable. Surprisingly, when cells are compromised for some but not all Cdc28-Cln kinase activity, Pho85 as well as Pcl1 and Pcl2 are essential.

In haploid yeast cells mutant for *cln1* and *cln2*, *PHO85* is essential. The same is seen with the cyclin counterparts to *PHO85*; *PCL1* or *PCL2* are essential in haploid yeast cells mutant for *cln1* and *cln2*. This co-dependency upon either the cyclins and the cyclin dependent kinase further supports the genetic interaction between Pho85 and the Pcl's. Additionally, in diploid yeast cells mutant for *cln1* and *cln2*, *PCL1* is essential. This

evidence suggests that when cells are deficient in Cdc28-Cln kinase activity Pho85-Pcl kinase activity becomes essential as seen by the lethality of the *cln1Δ cln2Δ pho85Δ and cln1Δ cln2Δ pcl1Δ pcl2Δ* haploid cells and the *cln1Δ cln2Δ pcl1Δ* diploid cells, respectively (Espinosa et al. 1994 and Measday et al. 1994). All these yeast mutants arrest in G1. There is also some in vivo data from Brenda Andrews lab that says there may be a total of eight or nine cyclin like molecules that associate with Pho85 (unpublished results). The function of any of these proteins is not known except for Pho80 which was found in the early 1970's by Oshima as a negative regulator of *PHO5* transcription and cyclin counterpart to Pho85 in the phosphate pathway.

The question now becomes "What possible functions may Pho85 have in cell cycle progression? Since we know that its kinase activity is needed in certain situations, is this of biological relevance? When Cdc28-Cln kinase activity is diminished is there a overlapping substrate specificity between Cdc28 and Pho85? The overlapping specificity would probably be accompanied by a higher Km because in normal situations the substrate would be phosphorylated by Cdc28 and not Pho85. However, a build up ofsubstrate due to a decline in Cdc28-Cln kinase activity may cause the interaction between certain substrates and Pho85. Also, it is possible that a parallel pathway may exist for activating transcription of certain proteins in G1 to progress through START which Pho85-cyclin kinase activity. It is also possible that Pho85-cyclin kinase activity could activate some bypass pathway in which certain proteins normally activated by Cdc28-Cln kinase activity are activated.

The above background information is the basis for this research. Pho85 has some role in cell cycle progression, specifically some role in G1 and passage through START and thus commitment to the cell cycle. However it is unclear what function Pho85 plays in cell cycle progression. I have attempted to design a genetic screen to identify substrates and downstream effectors of the Pho85-Pcl protein complexes. The following is a detailed account of the design of the genetic screen and the results achieved.

Materials and Methods

Strains

1397-19B: *MATα bar1 trp1 leu2 ura3 ade1 his2* - 15D background

1255-5C: *MAT<u>a</u> bar1 trp1 leu2 ura3 ade1 his2* - 15D background - J. McK. strain

1591-11A: *MATα cln1Δ cln2Δ leu2Δ::GAL1-CLN1 pcl1Δ::HIS3 pcl2Δ::URA3 trp1 leu2 ura3 ade1 his3* - 15D background

1591-1C: *MATα cln1Δ cln2Δ leu2Δ::GAL1-CLN1 trp1 leu2 ura3 ade1 his3* - 15D background

The above four strains were generously provided by Dr. Fred Cross

JO313-8C: *MAT<u>a</u> cln1 ∇TRP1 cln2 ∇LEU2 trp1 leu2 ura3 ade2 his3 lys2* - YPH274 background

JO313-1A: *MATα cln1 ∇TRP1 cln2 ∇LEU2 trp1 leu2 ura3 ade2 his3 lys2* - YPH274 background

Strains generated by Mating

GP4-1A: *MAT<u>a</u> cln1Δ cln2Δ trp1 leu2 ura3 his3 lys2*

Made by crossing 1591-1C (*MATα cln1Δ cln2Δ leu2Δ::GAL1-CLN1 trp1 leu2 ura3 ade1 his3*) with JO313-8C *(MAT<u>a</u> cln1 ∇TRP1 cln2 ∇LEU2 trp1 leu2 ura3 ade2 his3 lys2*).

The resulting diploid (only genes of selected interest)

MATa	*cln1∇TRP1*	*cln2∇LEU2*	*leu2*	*lys2*
MATα	*cln1Δ*	*cln2Δ*	*leu2Δ::GAL1-CLN1*	*LYS2*

After sporulation and dissecting tetrads from the resulting diploid - selected spores that were phenotypically leu-, trp1, lys- .

GP5-pcl2Δ *MATa cln1Δ cln2Δ pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2*

This strain was made by disrupting *MATa cln1Δ cln2Δ trp1 leu2 ura3 his3 lys2* with a *pcl2Δ::LYS2* disruption vector.

$pcl1_{ts}$ loop-ins *MATa cln1Δ cln2Δ PCL1:: $pcl1_{ts}$-BS-URA3 pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2*

This strain was made by integrating *$pcl1_{ts}$* in *MATa cln1Δ cln2Δ pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2* at the *PCL1* locus. The integration vector backbone Bluescript™ contained the *URA3* gene.

loop-outs *MATa cln1Δ cln2Δ $pcl1_{ts}$ pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2*

Made by plating *MATa cln1Δ cln2Δ PCL1:: $pcl1_{ts}$-BS-URA3 pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2* onto 5-FOA and selecting colonies that are viable at 25°C and inviable at 38.5°C.

Growth Conditions and Medias

All yeast were grown in YEPD, YEPG, or SD at 30°C unless testing for temperature sensitivity where 25°C, 37°C, or 38.5°C was used.

Experimental Procedures

A standard protocol was used for site directed mutagenesis of *PCL1* to make the corresponding mutation observed in the pho80 temperature sensitive allele. Single stranded DNA carrying the sense strand of *PCL1* was made and in vitro DNA synthesis was primed with a chemically-synthesized oligonucleotide carrying a base mismatch with the complementary sequence. The nucleotide sequence changed from TTA to TGA in the sense strand, corresponding to a leucine to serine substitution at amino acid position 144.

See below the General Strategy section which describes several different methods used in the mutagenesis of *PCL1*. The temperature sensitive alleles obtained were from random PCR mutagenesis and so the other methods are not described in detail.

Random PCR mutagenesis of *PCL1* was achieved by using protocols described by Fromant et al. 1995, Leung et al. 1989, and Rellos et al. 1993. PCR primers that bordered the entire open reading frame of *PCL1* including 5' and 3' flanking regions were used in DNA synthesis. The mutant library of mature PCR products (1528 bp) were either cloned directly into pRS314 *TRP1* vectors prior to yeast transformation, or at the time of transformation, a plasmid (pRS314 *TRP1* -SalI site in polylinker destroyed) carrying the *PCL1* open reading frame and promoter region digested with SalI and StuI creating a

gapped plasmid (Muhlrad et al. 1992) with 5' and 3' homology (177 bp 5' and 528 bp 3') to the *pcl1* mutant DNA's and the library of DNA's was transformed into yeast. Recombination between the homologous regions of the PCR product and the gapped plasmid created a intact plasmid now carrying the mutant *pcl1*.

All yeast transformations were standard lithium acetate transformations (Gutherie and Fink). The yeast strain used in all the transformations for screening temperature sensitive alleles of *PCL1* was 1591-11A: *MATα cln1Δ cln2Δ leu2Δ::GAL1-CLN1 pcl1Δ::HIS3 pcl2Δ::URA3 trp1 leu2 ura3 ade1 his3* - 15D background (See above in Strain section).

Construction of the final strain for use in the high-copy suppression screen: *MAT*a *cln1Δ cln2Δ pcl1* $_{ts}$ *pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2* was made by genetically manipulating strain 4-1A (see above in Strain section). 4-1A, *MAT*a *cln1Δ cln2Δ trp1 leu2 ura3 his3 lys2*, was first disrupted for *PCL2* using a *pcl2Δ::LYS2* disruption vector (provided by of Brenda Andrews). The entire open reading frame of *PCL2* was removed. This strain called GP5-pcl2Δ (*MAT*a *cln1Δ cln2Δ pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2*) was then used to loop in the *pcl1* temperature sensitive allele. The integration vector backbone was made by taking the 1.1 kB fragment from pJJ244, which contained the entire *URA3* gene, and filling in the ends with Klenow DNA polymerase. This blunted fragment was then inserted into the polylinker of Bluescript™ at the Ecl136II (blunt cutter) site. The resulting vector was called pBS-URA3. The #10 *pcl1* temperature sensitive mutant (See below) was then cloned into the XhoI/SpeI sites of pBS-URA3 in the polylinker. Partial digestion of this plasmid with HindIII preferentially destroyed the HindIII site in the polylinker and left the HindIII site at -120 in the promoter region. This

final integration vector was called pBS-pcl1$_{ts}$-URA3. Integration of the mutant was accomplished by digesting pBS-pcl1$_{ts}$-URA3 with HindIII and transforming GP5-pcl2Δ (*MATa cln1Δ cln2Δ pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2*) with the linear DNA. All integrations and disruption were checked by Southern analysis.

Temperature sensitivity was determined by plating yeast transformants and yeast mutants at 25°C and then replica plating at 37°C or 38.5°C. Colonies that grew at 25°C and did not grow at 37°C (and some cases 38.5°C) were temperature sensitive. In some cases where the characterization *of MATa cln1Δ cln2Δ pcl1 $_{ts}$ pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2* was in question, the transformants were plated directly at the non-permissive temperature.

Plasmid Constructs and Synthetic Oligonucleotides

Bacterial Plasmid Constructs

pBluescript™ Used to make integration vector

pJJ244 *URA3* containing plasmid - used to make integration vector.

pFHE27: Bluescript™ carrying *PCL1* orf and promoter regions. f1 origin of replication for single strand DNA production. DNA template for PCR reactions-provided by F.H. Espinoza.

pBS-pcl1$_{ts}$-URA3

This plasmid was used to integrate the *pcl1 $_{ts}$* allele into *MATa cln1Δ cln2Δ pcl1 $_{ts}$ pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2*

Yeast Plasmid Constructs

pBA616 A SalI-BglI fragment of yeast genomic DNA containing the *PCL2* ORF excised from plasmid pRB534 (JCB v.114 p. 443) and inserted into the Sal1/BamHI sites of pUC18. This plasmid was used to make probes to check *pcl2Δ::LYS2* disruption.

pBA732 *pcl2Δ::LYS2* disruption vector; pUC18 vector backbone.

Note: Both pBA vectors were graciously provided by Brenda Andrews.

Yeast Plasmids used in Screening for Temperature Sensitive alleles of *PCL1*

pRS314 ARS/CEN *TRP1* vector

pRS314* ARS/CEN *TRP1* vector ; Sal1 site in the polylinker destroyed.

Yeast Plasmids used for Characterization of the yeast mutants containing *pcl1ts* :

Testing Suppression of the temperature sensitive phenotype.

pRSPCL1 ARS/CEN *PCL1* (Vector control - pRS314)

2μ-CLN1 High Copy *CLN1*

YEp13 Yeast episomal plasmid - *LEU2* selectable marker

2μ-CLN2 High Copy *CLN2*

YEp24 Yeast episomal plasmid - *URA3* selectable marker

Synthetic Oligonucleotides

Site directed Mutagenesis:	5'-CCAGTTCAACAGTTGACTCAACTGCCTTTC-3'
	Anneals to the sense strand of *PCL1* at position +418
	to position +447.
PCR Mutagenesis: Primer 1	5'-CGTGTTAAAAATGCGTCCGGCGCG-3'
Primer 2	5'-CGCAGCTCGAGGTTCGCACTACGATACTTTGC-3'

Design and Strategy of Genetic Screen/Selection

It is thought that cyclins may provide substrate specificity for their cdk partners. I hypothesize that creating a temperature sensitive mutant of a cyclin that associates with Pho85 in the context of the synthetic lethality observed when yeast are deficient in Cdc28-Cln kinase activity may provide a tool for identifying substrates or downstream effectors of the Pho85-cyclin complexes. This may elucidate its role in cell cycle progression.

A haploid yeast strain mutant for *cln1Δ cln2Δ pcl1Δ pcl2Δ* is inviable (Measday et al. 1994). Creating a strain that is temperature sensitive for *PCL1* in the same background where the synthetic lethality is seen produces the following: *cln1Δ cln2Δ* $\boldsymbol{pcl1_{ts}}$ *pcl2Δ*. This strain is viable at the permissive temperature and inviable at the non-permissive temperature. Using a high copy library, one can high copy suppress the synthetic lethality at the non-permissive temperature. The plasmids that suppress the lethality may contain genes that code for substrates or downstream effectors of the Pho85-Pcl complex.

General Strategy

Step One: Mutagenesis of *PCL1* ——————→ Possible Methods

a) Random PCR Mutagenesis

b) Bacterial Mutator Strain

c) Hydroxylamine

d) Site Directed Mutagenesis

Step Two: Screen for temperature sensitive alleles of *PCL1*

Allows growth of a *MAT α cln1Δ cln2Δ pcl1Δ pcl2Δ leu2Δ::LEU2-GAL1-CLN1* strain on glucose at 25°C but not at 37°C.

Step Three: Construction of *cln1Δ cln2Δ* $pcl1_{ts}$ *pcl2Δ::LYS2* strain.

Step Four: High Copy Suppression Screen/Selection for plasmids containing genes that allow the temperature sensitive mutant, *MAT a cln1Δ cln2Δ* $pcl1_{ts}$ *pcl2Δ::LYS2*, to grow at 38.5°C.

Results and Discussion

Mutagenesis of *PCL1*

There were several ways to mutagenize *PCL1* and obtain a temperature sensitive allele, but I tried the most obvious way first. Temperature sensitive alleles of *PHO80* had been isolated (Lemire et al., 1985) and one such mutant was characterized and found to be a T → C transition at position 488 of the *PHO80* coding sequence (Madden et al., 1988). Both *PCL1* and *PCL2* have homology with this region that produced the temperature sensitive allele of *PHO80*.

Protein alignment of homologous regions in *PCL1*, *PCL2*, and *PHO80*:

PCL1 PST I HRI FLACL ILSAKFHNDSSPLNKHWARYTDGL FTLED INLMERQL **L** QLLNWDL

PCL2 ETTRHRI FLGCL ILAAKTLNDSSPLNKHWAEYTDGL L ILREVNTI ER EL **L** EYFDWDV

PHO80 SLTAHRFLLTATTVATKGLCDSFSTNAHYAKV----GGVRCHELNI LEND F **L** KRVNYR I

↑

This residue in the *PHO80* ts mutant is ⟶ **S**

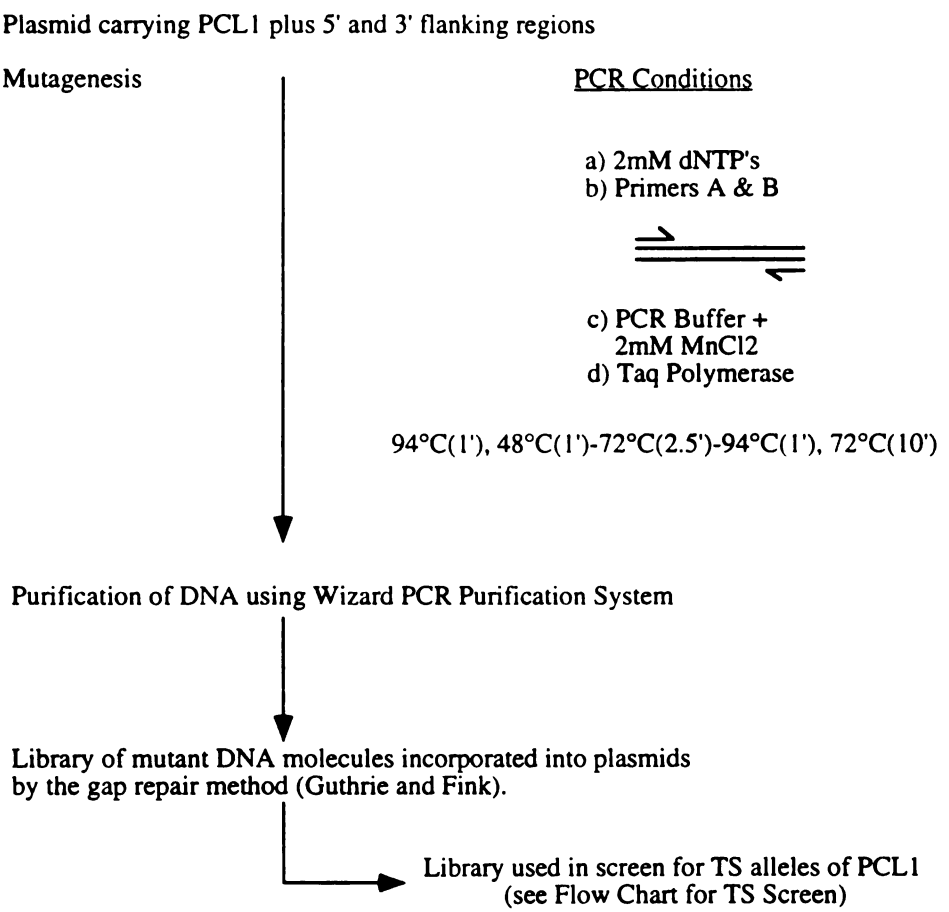
I therefore made the corresponding mutation (leucine → serine) in the *PCL1* coding sequence on a ARS/CEN plasmid and transformed this plasmid into a *MATα cln1Δ cln2Δ pcl1Δ pcl2Δ leu2Δ::LEU2-GAL1-CLN1* strain (1591-11A, see Materials and

Methods). The plasmid was not temperature sensitive as seen by the growth of the tester strain on glucose at the permissive and non-permissive temperatures.

Note: The tester strain,1591-11A, was obtained from Fred Cross and is inviable on glucose because the GAL1 promoter is repressed in glucose.

I then tried a number of in vivo and in vitro mutagenic protocols. Random PCR mutagenesis proved to be successful. The concept behind random PCR mutagenesis is to provide mutagenic conditions that increase the inherent mutagenic rate of Taq™ polymerase. The normal mutagenic rate for Taq™ polymerase is ~ 0.1%. By the addition of manganese chloride to 2mM, the mutagenic rate increased to ~1-2%. Figure 1 describes the details of the random PCR mutagenesis.

Figure 1: RANDOM PCR MUTAGENESIS

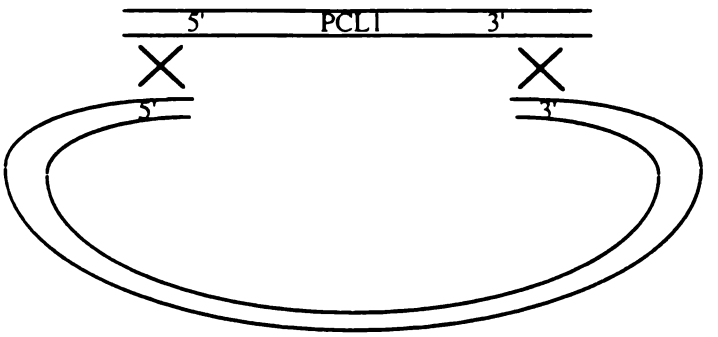


Screening for *PCL1* Temperature Sensitive Mutants

The library of mutant *PCL1* DNAs were then screened for temperature sensitivity. The tester strain used to screen the mutants is Fred Cross strain 1591-11A, *MATα cln1Δ cln2Δ pcl1Δ::HIS3 pcl2Δ::URA3 leu2Δ::LEU2-GAL1-CLN1 ura3 his3 trp1*. A gapped plasmid protocol was used (Guthrie and Fink). This method takes advantage of the recombination machinery that yeast have. By transforming yeast with the library of mutant DNA's and a gapped ARS/CEN plasmid, pRS314*, carrying 5' and 3' homology to the DNA's, recombination can occur. The yeast will recombine the gapped plasmid and the PCL1 mutants to give a completed plasmid. The recombinant plasmid is only selected which contains the gapped plasmid carrying the selectable marker *TRP1*, and the mutant *PCL1* which can confer viability to the base strain at the permissive temperature. This type of screen provided two stages of stringency by requiring the *TRP1* marker and a *PCL1* mutant that is at least active at the permissive temperature, 25°C. Screening temperature sensitive mutants is described in Figure 2:

Figure 2: SCREENING FOR PCL1 TEMPERTURE SENSITIVE MUTANTS

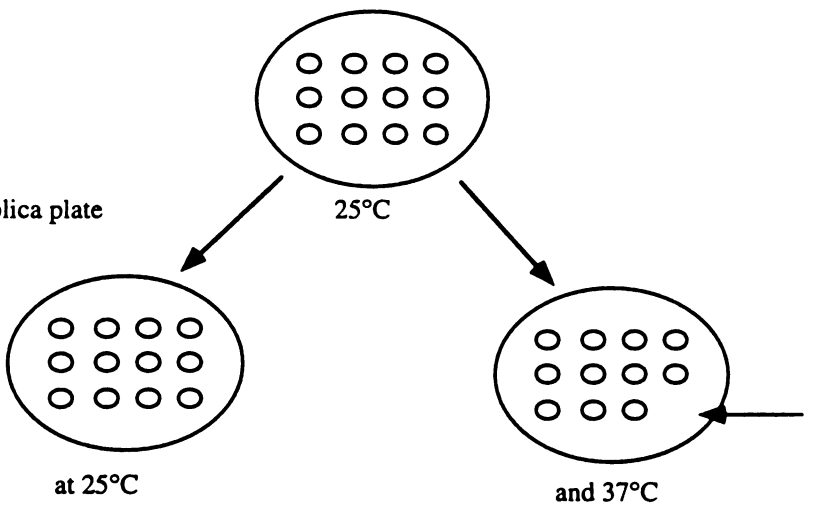
1. Transform the library of mutant PCL1 DNA obtained by random PCR mutagenesis and a gapped plasmid that has 5' and 3' homology with PCL1 into a *cln1Δ cln2Δ pcl1Δ::HIS3 pcl2Δ::URA3 leu2::LEU2-GAL1-CLN1 trp1 strain.



pRS314 ARS/CEN TRP1 vector backbone

2. Plate transformation onto SD-TRP plates and place at RT.

3. Replica plate



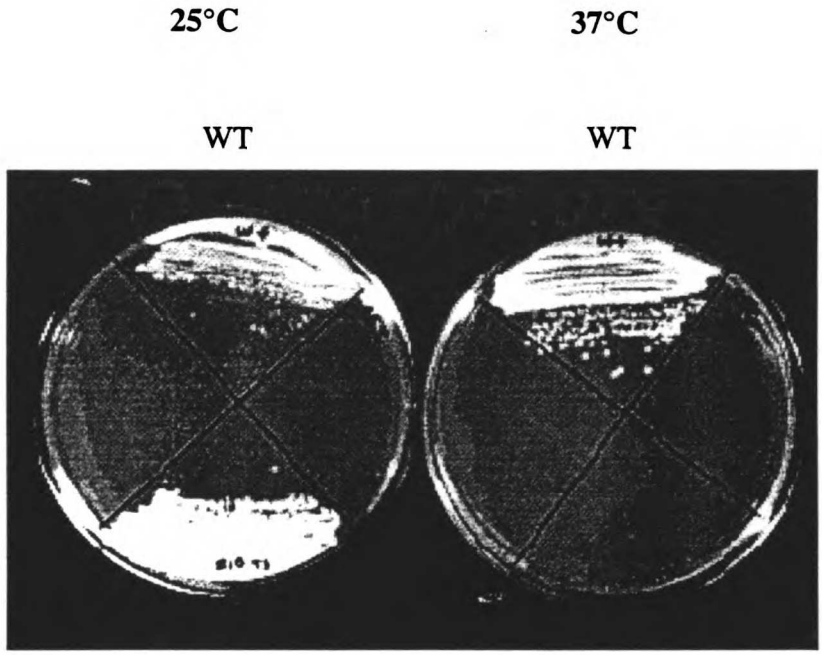
4. Select the colonies that grow at 25°C but not at 37°C.

	Growth on SD	
	25°C	37°C
*Base strain		
Base strain + PCL1	+	+
Base strain + $pcl1_{ts}$	+	—

Putative temperature sensitive mutants were obtained. These plasmids were rescued from the yeast and retransformed into the base strain. One plasmid, #10 *pcl1*$_{ts}$, retested and conferred temperature sensitivity to the tester strain. See Figure 3 below.

Figure 3: **Temperature Sensitive Phenotype of a** PCL1 **Mutant obtained by Random Mutagenesis by PCR**

For details on how the screening for these ts mutants was done see flow chart : Screening for PCL1 Temperature Sensitive Mutants (Figure 2).

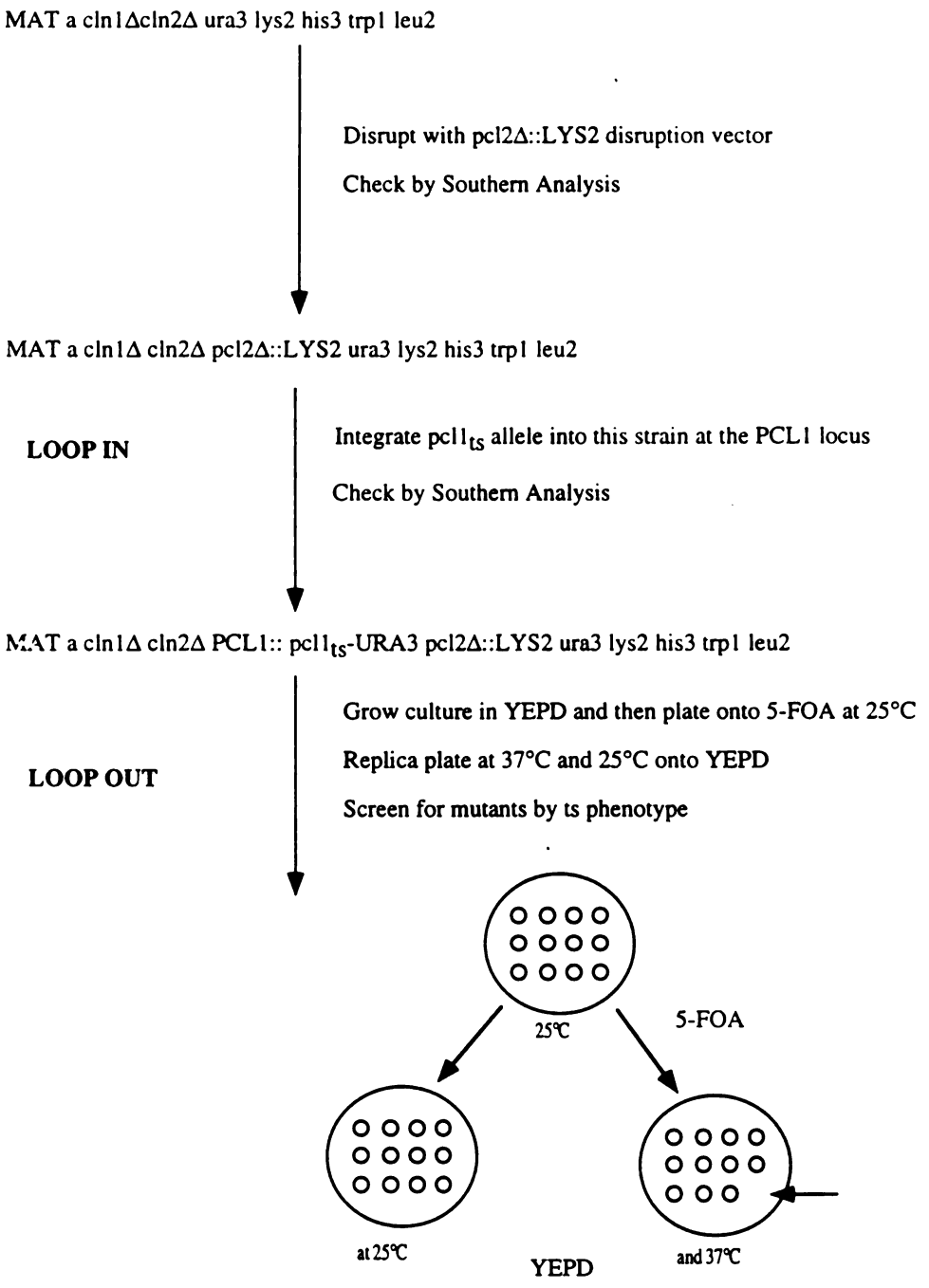


MATa cln1Δ cln2Δ pcl1Δ::HIS3 pcl2Δ::URA3 leu2::LEU2-GAL1-CLN1 ura3 trp1 his3 [pRS-pcl1$_{ts}$-TRP1]

Construction of Strain for the High Copy Suppression Screen

Now that a putative temperature sensitive allele of *PCL1* was made, it had to be integrated into the proper strain background in order to proceed with the high copy suppression screen and/or selection. The following flow chart gives step by step details of how the final strain, *MAT a cln1Δ cln2Δ* ***pcl1$_{ts}$*** *pcl2Δ::LYS2 ura3 lys2 his3 trp1 leu2*, was made.

Figure 4: **CONSTRUCTION OF STRAIN FOR THE HIGH COPY SUPPRESSION SCREEN**



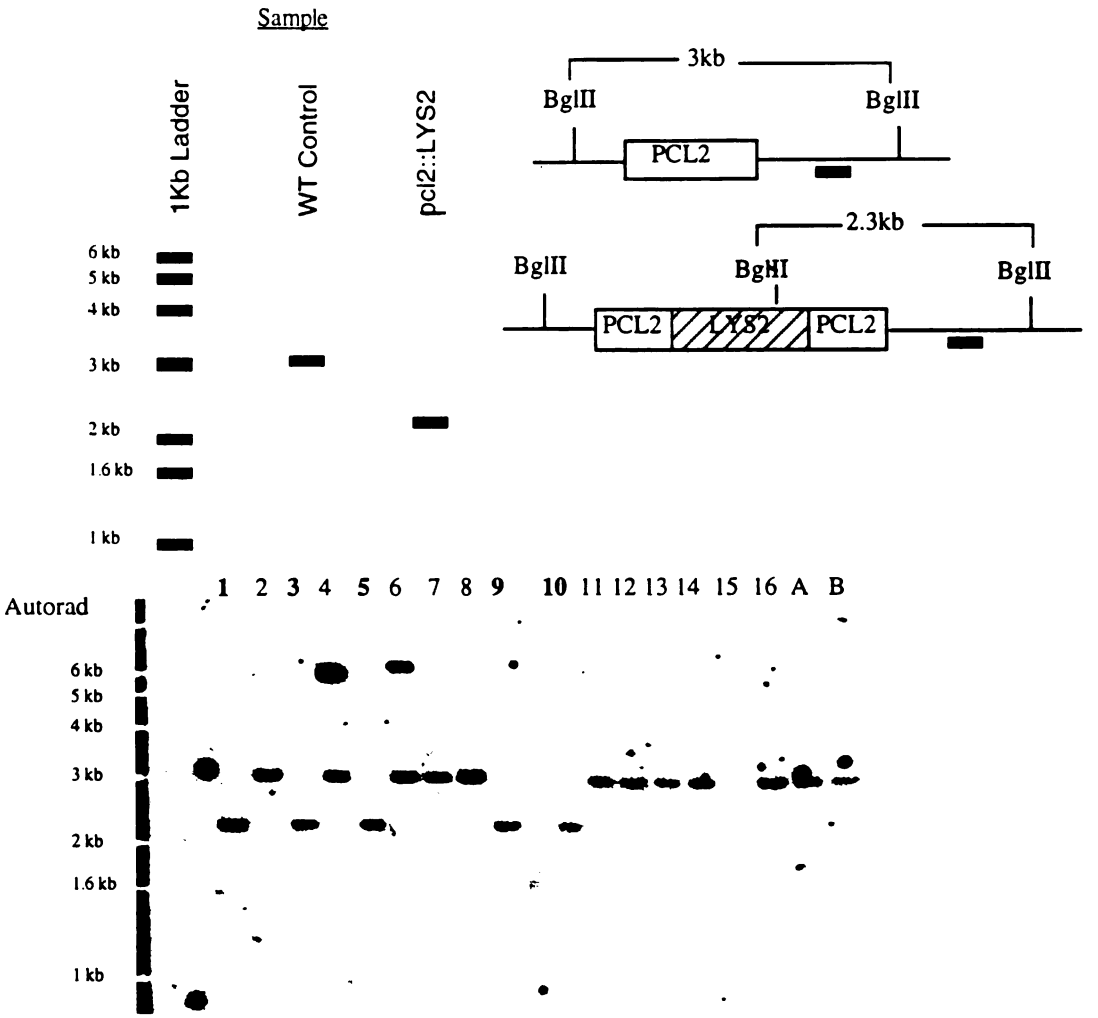
The starting strain in this construction scheme is GP4-1A. This strain was made by a mating reaction and dissection of tetrads. The information is described in the "Materials and Methods" section. The next step was to disrupt *PCL2* with the *pcl2Δ::LYS2* disruption vector, pBA732. As noted in the flow chart, after each genetic manipulation, a check was done by Southern analysis.

Using a 600 bp XbaI fragment of the 3' region of *PCL2* from pBA616 (See "Materials and Methods") I probed a Southern blot of genomic DNA from the yeast transformants to check the *pcl2Δ::LYS2* disruption. A schematic and picture of the autoradiograph is shown in Figure 5.

Figure 5: Southern Analysis for Diagnosis of pcl2Δ::LYS2 Disruption

Selected LYS+ yeast transformants after transformation with pcl2Δ::LYS2 disruption vector. Digested genomic DNA with BglII.

Schematic:



Bold = Proper Disruption

#= samples
A & B= wt controls

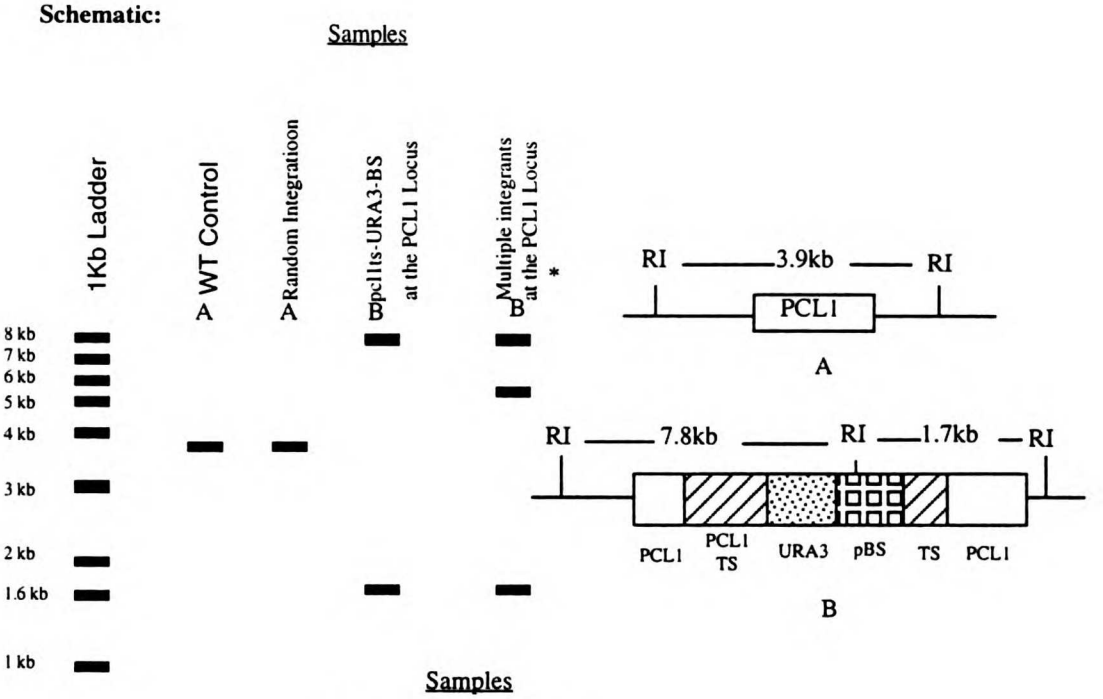
If properly disrupted a ~2.3 kB band should be present and wt should have a ~3 kB band. This is shown above in the schematic.

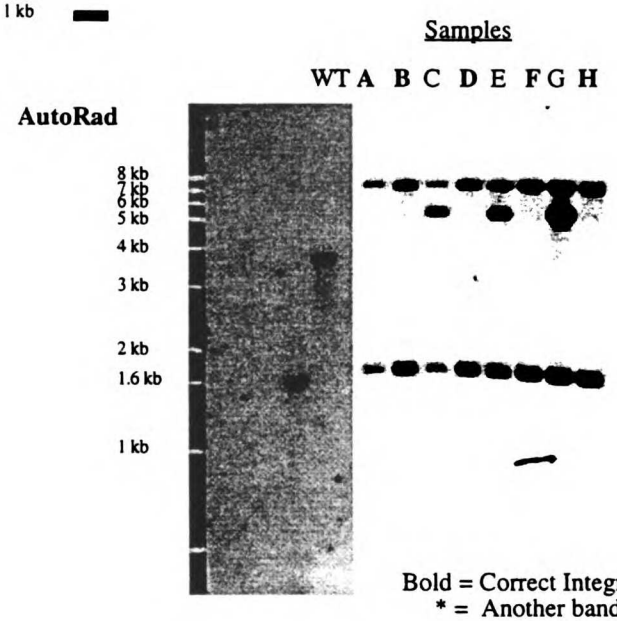
After disrupting *PCL2*, I had with the following strain: GP5-pcl2Δ, *MATa cln1Δ cln2Δ pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2*. I now had to integrate (loop-in) the #10 *pcl1* temperature sensitive mutant. The integration vector pBS-pcl1$_{ts}$-URA3 was digested with HindIII and GP5-pcl2Δ (*MATa cln1Δ cln2Δ pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2*) was transformed with this digested DNA. The yeast were plated onto SD-URA at 25°C. Eight URA+ transformants (A-H) were selected and checked by Southern analysis. I probed the Southern blot of genomic DNA from the integrants digested with EcoRI with an internal 604 bp HindIII/PstI fragment. This probe hybridizes to the first 484 bp of the coding region and 120 bp of the promoter region (i.e. -120 to +484). A schematic and picture of the autoradiograph is shown in Figure 6.

Figure 6:Southern Analysis for Diagnosis of pBS-pcl1$_{ts}$-URA3 Integration

Selected URA+ yeast transformants after transformation with pcl1$_{ts}$-URA3 integration vector. Digested genomic DNA with EcoRI.

Schematic:



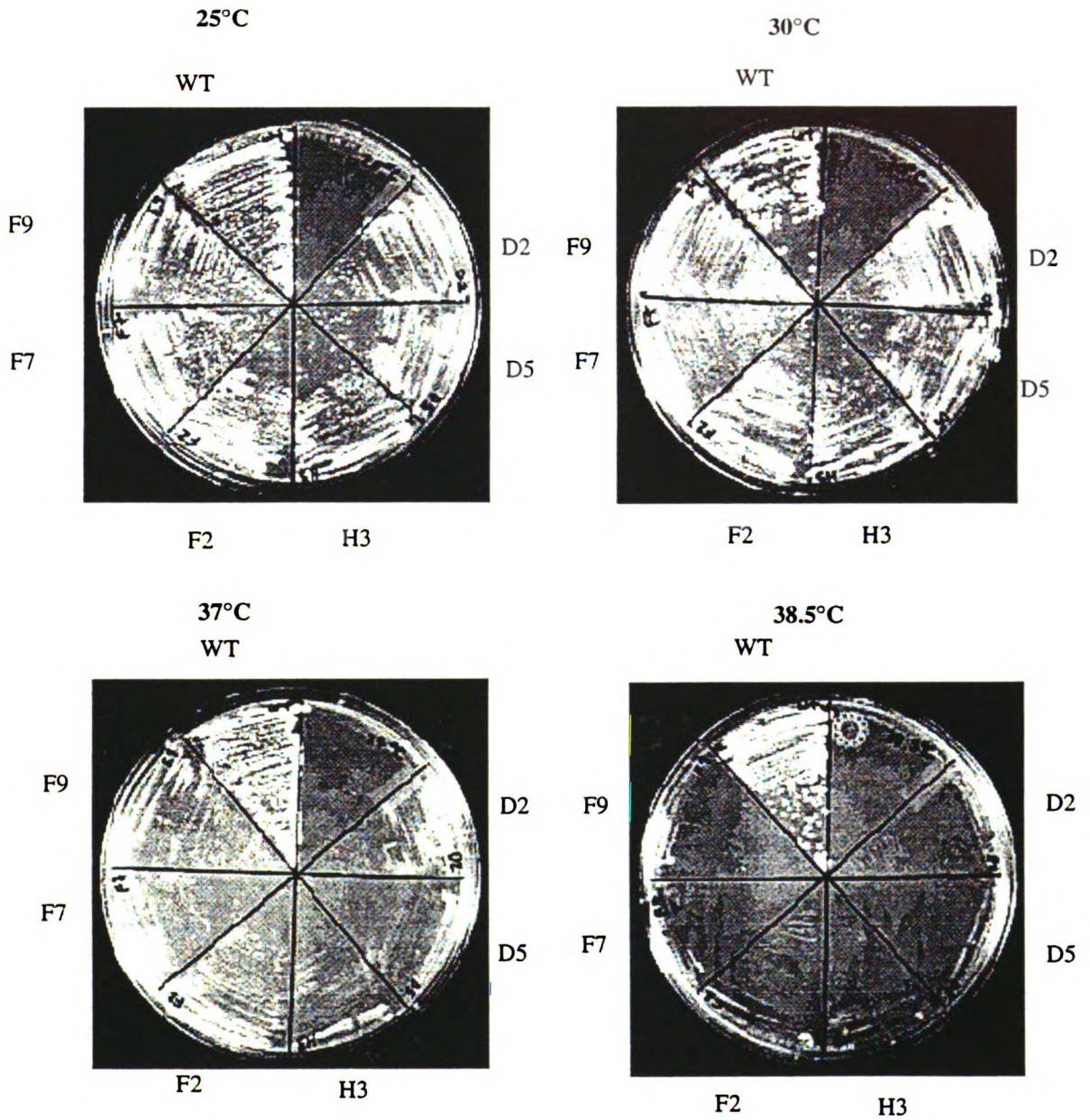


Bold = Correct Integration
* = Another band(s) should appear on autorad.

The schematic describes the three different possible ways the vector could integrate as per Southern analysis: random integration in the genome, single integration at the *PCL1* locus, and multiple integrations at the *PCL1* locus. Of the eight tested, five were correctly integrated once at the *PCL1* locus and the other three had multiple integrations at the *PCL1* locus.

I grew YEPD cultures of the five correct integrants. Recombination between the wt and mutant *PCL1* DNA regions caused the looping out of either the wt type or mutant DNA. Plating these yeast onto 5-FOA assured that a recombination event took place between the wt and mutant *PCL1* DNA's because yeast still expressing the *URA3* gene product were inviable due to the toxicity of 5-FOA. The yeast were plated at 25°C and as previously shown in Figure 5, the temperature sensitive mutants were selected by growth at 25°C but not at 37°C. There was some slight growth at 37°C but increasing the temperature to 38.5°C caused a more complete growth arrest. The mutants D2, D5, F2, F7, F9, and H3 (*MATa cln1Δ cln2Δ pcl1* $_{ts}$ *pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2*) all showed a severe temperature sensitivity at 38.5°C. WT showed no growth defect at 38.5°C. Figure 7 is picture of these mutants streaked out for singles on YEPD at 25°C, 30°C, 37°C, and 38.5°C.

Figure 7: **TS Phenotype of MAT a cln1Δ cln2Δ $pcl1_{ts}$ pcl2Δ::LYS2 ura3 lys2 his3 trp1 leu2**



Characterization of the Temperature Sensitive Mutant

The temperature sensitive allele of *PCL1* was incorporated into the genetic background *MATa cln1Δ cln2Δ pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2 creating MATa cln1Δ cln2Δ pcl1 $_{ts}$ pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2*. At 38.5°C this yeast mutant now behaves like the quadruple delete, *cln1Δ cln2Δ pcl1Δ pcl2Δ*, by arresting its growth. However, its ability to grow at 25°C allows one to try and understand the function of the Pho85-Pcl1 protein complex by finding suppressors of the synthetic lethality at 38°C.

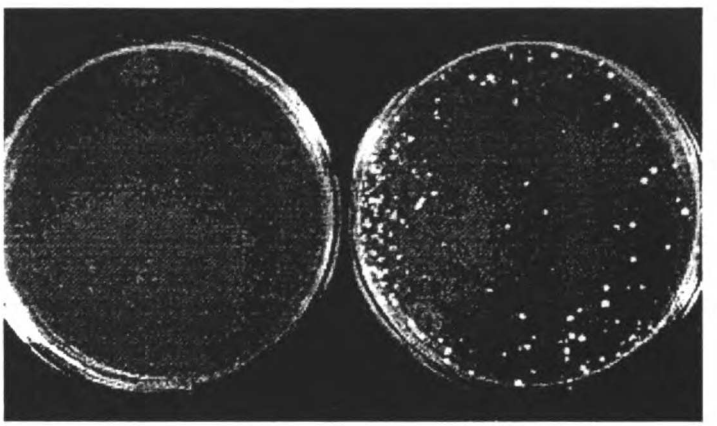
An hypothesis can be made that the wt activity of Cln1, Cln2, Pcl1, and Pcl2 could rescue the mutant *MATa cln1Δ cln2Δ pcl1 $_{ts}$ pcl2Δ::LYS2 trp1 leu2 ura3 his3 lys2* at the non-permissive temperature. To simulate the suppression screen, which I would assume to retrieve high copy *CLN1*, *CLN2*, *PCL1*, and *PCL2*, I transformed two of the mutants (D2 and D5) with certain ARS/CEN and 2μ plasmids. I transformed D2 and D5 with the following plasmids:

pRSPCL1	ARS/CEN *PCL1*
pRS314	ARS/CEN vector control - *TRP1* selectable marker
2μ-CLN1	High Copy *CLN1*
YEp13	Yeast episomal plasmid - *LEU2* selectable marker
2μ-CLN2	High Copy *CLN2*
YEp24	Yeast episomal plasmid - *URA3* selectable marker

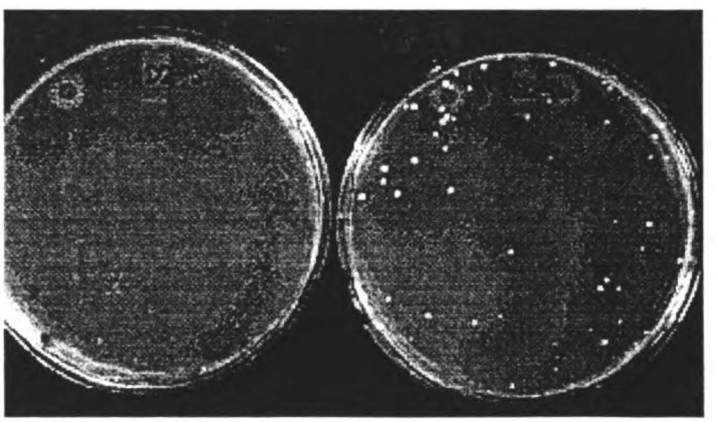
Figures 8 and 9 are pictures of the transformation plates showing suppression of the lethality of mutants D2 and D5, respectively, by pRSPCL1 and 2μ-CLN1 at 38.5°C. Vector controls show that the suppression is specific to the activity of Pcl1 and Cln1.

Figure 8: Suppression of Temperature Sensitive Phenotype at 38.5°C by Known G1 Cyclins

Transformation of muatant D2 with ARS/CEN of 2μ plasmids carrying genes encoding G1 cyclins. Transformation plated directly at 38.5°C.



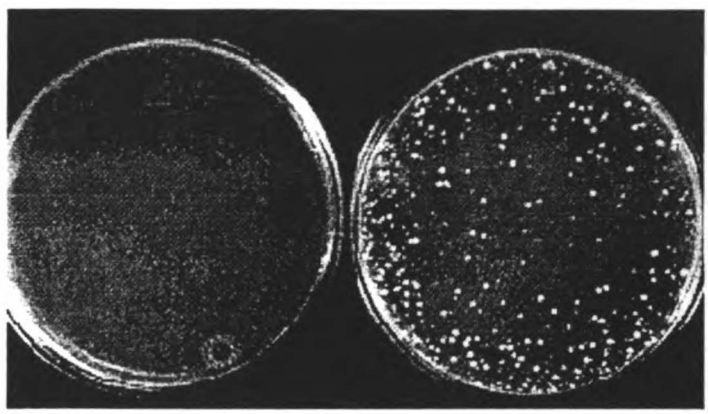
pRS314 pRSPCL1 (ARS/CEN)



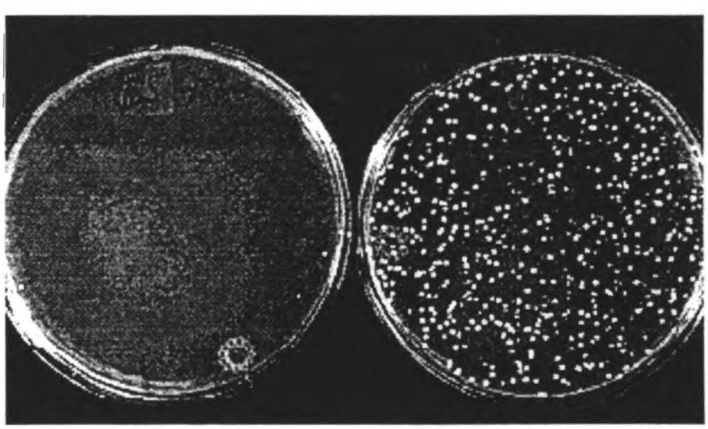
YEp13 2μ-CLN1

Figure 9: Suppression of Temperature Sensitive Phenotype at 38.5°C by Known G1 Cyclins

Transformation of mutant D5 with ARS/CEN and 2μ plasmids carrying genes encoding G1 cyclins. Transformation plated directly at 38.5°C.



pRS314 pRSPCL1 (ARS/CEN)



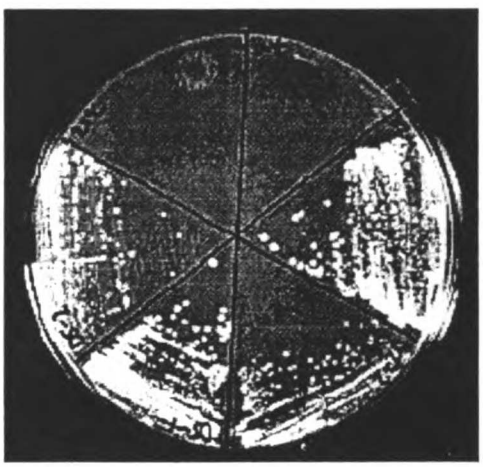
YEp13 2μ-CLN1

In Figures 10, 11, and 12, I show suppression of temperature sensitive lethality of the mutants D2 and D5 by ARS/CEN *PCL1*, 2μ-*CLN1*, and 2μ-*CLN2*, respectively. Transformants were streaked for singles from the 25°C plates onto the appropriate SD minus required amino acid (for selection of plasmid) plate at 38.5°C. Formation of single colonies shown in the pictures (Figures 10, 11, and 12) show that the ARS/CEN *PCL1*, 2μ-*CLN1*, and 2μ-*CLN2* plasmids rescues the temperature sensitive lethality at 38.5°C. This suppression is specific to these cyclins because the vector controls (pRS314, YEp13, and YEp24) do not suppress the temperature sensitive phenotype.

Figure 10: Suppression of Temperature Sensitive Phenotype at 38.5°C by Known G1 Cyclins

Streak of mutants D2 and D5 with **ARS/CEN PCL1** and vector only

25°C



38.5°C

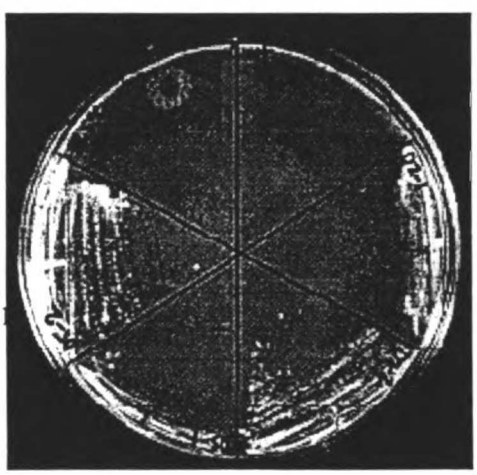
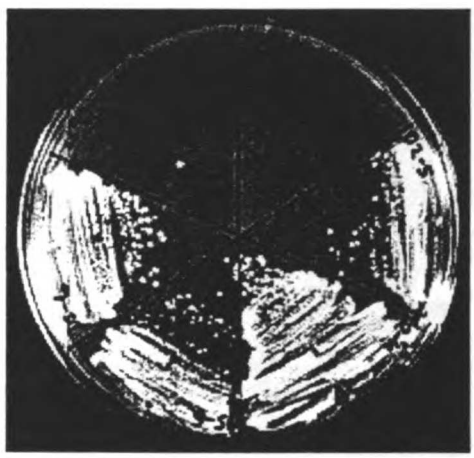


Figure 11: Suppression of Temperature Sensitive Phenotype at 38.5°C by Known G1 Cyclins

Streak of mutants D2 and D5 with **2μ-CLN1** and vector only

25°C



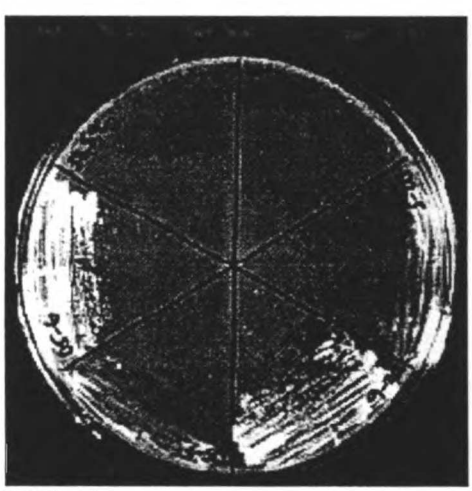
D5-2μ CLN1

D2-YEp13

D5-YEp13

D2-2μ CLN1

38.5°C



D5-2μ CLN1

D2-YEp13

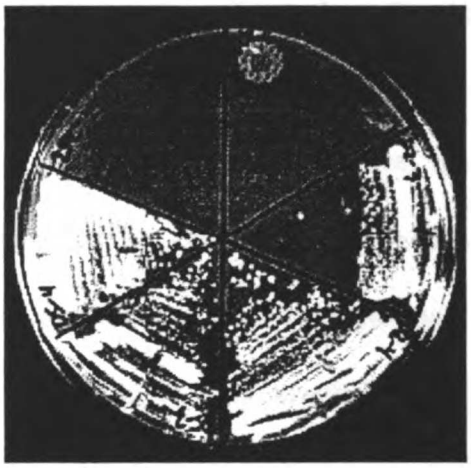
D5-YEp13

D2-2μ CLN1

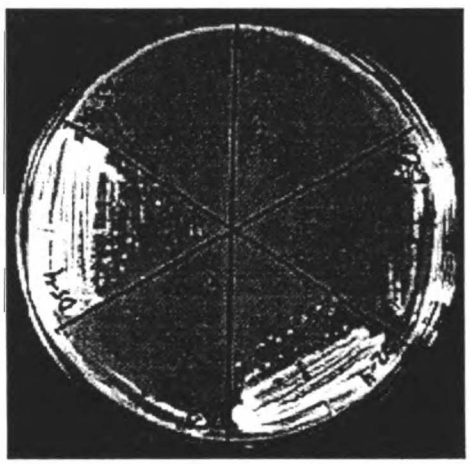
Figure 12: Suppression of Temperature Sensitive Phenotype at 38.5°C by Known G1 Cyclins

Streak of mutants D2 and D5 with **2μ-CLN2** and vector only

25°C



38.5°C



Conclusions

Cyclin-dependent kinases play a major role in cell cycle progression. They are the "engines" of the cell cycle. Many "switches" turn these "engines" on and thereby drive the proliferation of cells. The case is clearly seen in *S. cerevisiae*. Cdc28, the single most important engine, must operate at a sufficient level or cells arrest at particular stages in the cell cycle as seen with the G1 arrest of a *cln1Δ cln2Δ cln3Δ* mutant. The observation that cells mutant for *cln1Δ cln2Δ* are viable but *cln1Δ cln2Δ pho85Δ* and *cln1Δ cln2Δ pcl1Δ pcl2Δ* are inviable, says that another cdk, Pho85, may have a role in cell cycle progression. How does a protein previously shown to have function in phosphate regulation tie into the cell cycle? Are there cues from the environment that are relayed to the cell cycle machinery by Pho85 and its associated cyclins? The focus of my thesis was to elucidate what roles Pho85 and Pcl1 and Pcl2 has in cell cycle progression , specifically passage through G1.

I was interested in substrates or downstream effectors of the Pho85-Pcl protein kinase complexes. Knowing that cyclins may provide substrate specificity to their cdk partner, I decided to make a temperature sensitive allele of *PCL1*. This was accomplished by random PCR mutagenesis. I incorporated this temperature sensitive allele into a genetic background which made viability dependent upon the activity of the temperature sensitive allele at a permissive temperature (*MATa cln1Δ cln2Δ pcl1* $_{ts}$ *pcl2Δ::LYS2 trp1*

leu2 ura3 his3 lys2). I showed that known cyclins did in fact suppress the temperature sensitive lethality in both high and low copy plasmids.

I was not however able to proceed with the actual suppression screen. This would have included transforming the mutant with a high copy library and selecting for viability at 38.5°C. Rescuing and retesting the plasmids to make sure the interaction is real would be the next step. After a group of plasmids were retrieved in the screen/selection determination the identity of the genes on the plasmids would follow. One could eliminate all known cyclins by bacterial colony hybridization with ^{32}P labeled cyclin fragments. I would expect to obtain *CLN1*, *CLN2*, *CLN3*, *PCL1*, *PCL2*, and *PHO85* in my screen. In fact, retrieving these genes in the screen is an internal check that the screen works and the library used is fully representative of the known genes that should suppress the temperature sensitive phenotype. Of the plasmids left, I would then categorize the plasmids that suppress the *cln1Δ cln2Δ cln3Δ* mutant vs. the *cln1Δ cln2Δ pcl1 $_{ts}$ pcl2Δ* mutant. Some biochemistry would be done on the suppressor that may be substrates of the Pho85-Pcl kinase. Hopefully this work can be continued and eventually it may shed light on Pho85's role in cell cycle progression.

References and Acknowledgments

1. F. Hernan Espinoza*, Joseph Ogas, Ira Herskowitz, David O. Morgan; "Cell Cycle Control by a Complex of the Cyclin HCS26(PCL1) and the Kinase PHO85" *Science* vol. 266 p.1388(1994)

2. Vivien Measday, Lynda Moore, Joseph Ogas, Mike Tyers, Brenda Andrews*; " The PCL2 (ORFD)-PHO85 Cyclin-Dependent Kinase Complex: A Cell Cycle Regulator in Yeast" *Science* vol. 266 p.1391 (1994)

3. Kim Nasmyth (review) "Control of the yeast cell cycle by the Cdc28 protein kinase" *Current Opinion in Cell Biology* vol. 5:166-179 (1993)

4. Arie Kaffman, Ira Herskowitz, Robert Tijan, and Erin K. O'Shea*; Phosphorylation of the Transcription Factor PHO4 by a Cyclin-CDK Complex, PHO80-PHO85" *Science* vol. 263 p. 1153-1156 (1194)

5. Elizabeth O'Neill, Erin K. O'Shea* (submitted)

6. Michael Fromant, Sylvain Blanquet, and Pierre Plateau; " Direct Random Mutagenesis of Gene-Sized DNA Fragments Using Polymerase Chain Reaction" *Analytical Biochemistry* vol. 224 p. 347-353 (1995)

7. David W. Leung, Ellson Chen, David V. Goeddel; " A Method fro Random Mutagenesis of A Defined DNA Segment Using a Modified Polymerase Chain Reaction" *J. of Methods in Cell and Molecular Biology* vol. 1 No. 2 p. 11-15 (1989)

8. Peter Rellos and Robert K. Scopes; "Polymerase Chain Reaction-Based Random Mutagenesis: Production and Characterization of Thermostable Mutants of *Zymomonas mobilis* Alcohol Dehydrogenase-2" *Protein Exression and Purification* vol. 5 p. 270-277 (1994)

9. Denise Muhlrad, Rosalyn Hunter, Roy Parker* " A Rapid Method for Localized Mutagenesis of Yeast Genes" *Yeast* vol. 8 p.79-82 (1992)

10. Lemire, J. M, Wilcocks, T., Halvorson, H. O, and Bostain, K. A.; "Regulation of repressible acid phosphatase gene transcription in *Saccharomyces cerevisiae*" Mol. Cell.Biology vol. 5 p.2131-2141 (1985)

11. Madden, S. L., Creasy, C. L., Srinivas, V., Fawcett, W., and Bergman, L.W; "Structure and Expression of the *PHO80* gene of *Saccharomyces cerevisiae*" *Nucleic Acids Research* vol. 16 p.5959-5957 (1988)

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