ANALYSIS OF GENES THAT AFFECT TELOMERE STABILITY IN *YKU70* MUTANTS AND NEW APPROACHES FOR SEPARATION OF YEAST NUCLEIC ACIDS

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ANALYSIS OF GENES THAT AFFECT TELOMERE STABILITY IN *YKU70* MUTANTS AND NEW APPROACHES FOR SEPARATION OF YEAST NUCLEIC ACIDS

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I first want to thank my family for all of their support and patience with me throughout my life. Without them I would not be where I am today. I also want to thank my research advisor, Dr. Lewis, for being my mentor over the past two years. His dedication to his students far surpassed any expectation I had before entering this program. I will never forget everything he has done for me and no words will ever express my gratitude. Joaleah San Miguel, thank you for teaching me how to love chemistry and thank you for being my friend.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

CHAPTER I

INTRODUCTION

The yeast *Saccharomyces cerevisiae* is commonly used by scientists as a model organism for higher eukaryotes. Several reasons make strains of this yeast advantageous to biochemistry or genetic studies, including a rapid growth rate, a well-characterized genome, stable haploid and diploid states, and dynamic DNA transformation abilities. Genetic cloning and genetic engineering methods can be employed in yeast with particular ease due to advancement in DNA transformation techniques; plasmids can be incorporated into yeast either by direct integration into the genome or by an independent, replicating molecule. Unlike other organisms, transforming DNA through integration into the yeast genome follows the homologous recombination pathway. Thus, specific foreign DNA sequences can be incorporated at desired locations within the genome and creating gene knockouts is highly efficient. This fact has enabled researchers to study functions of genes and proteins of yeast *in vivo* (1).

Chromosomal stability is vital for cell survival and proliferation. In addition to the coding and intergenic regions of linear eukaryotic chromosomes, there are extensive noncoding regions at the ends of the DNA, which are comprised of a subtelomere and telomere. In *S. cerevisiae,* the subtelomere contains a short $(TG)_{1\text{-}3}$ DNA sequence that is flanked by the X region and Y' element, which is closer to the end of the chromosome (2). Both the X region and Y' element possess an autonomously replicating sequence (ARS) or origin of DNA replication (3). In contrast to the telomere, the DNA of the

subtelomere is compacted into nucleosomes (4). The telomeres are ~350 bp long and contain $(TG)_{1-3}$ repeat sequences with a G-rich 3' overhang (Figure 1) (5).

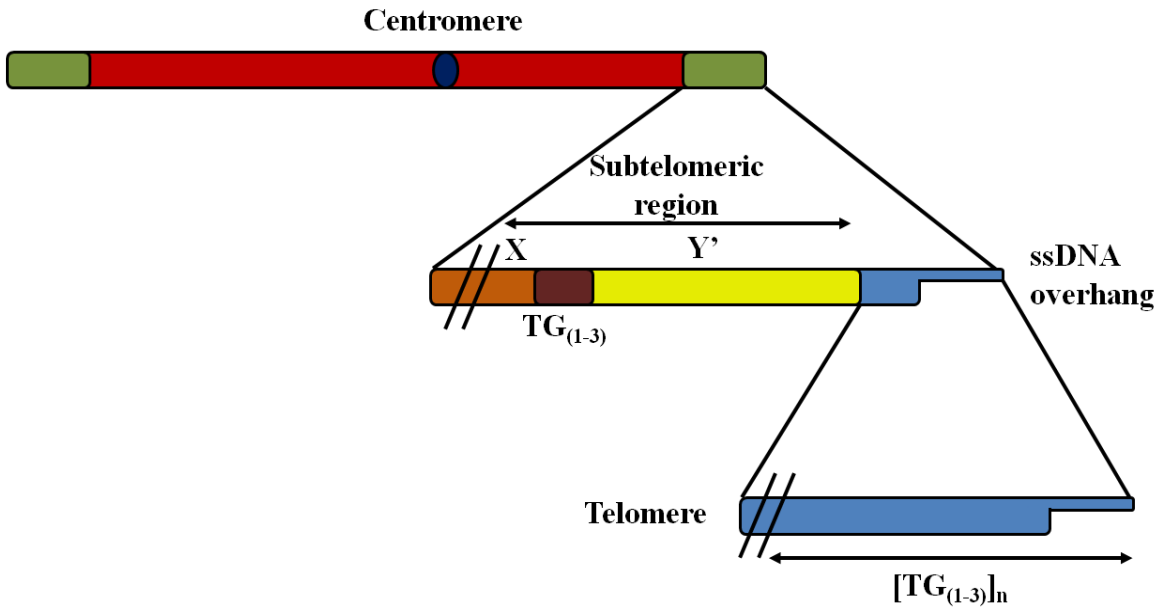

Figure 1. Illustration of the subtelomere and telomere within a linear chromosome of the budding yeast *S. cerevisiae*.

Telomeres represent or resemble double-strand break (DSB) ends. However, they do not fuse together, nor do they fuse to another DSB end located internally on the chromosome. This is due to the specialized DNA-protein complex located at the telomere, which enables the cell to distinguish a natural DSB end from the end of the chromosome. If the telomeres were to ever fuse together, this would be catastrophic to the cell because dicentric chromosomes (with 2 centromeres) could form that are unstable. The DNA-protein complex serves to cap the ends of chromosomes, thus, preventing any DNA end-joining reactions from occurring (6). Also, the cap structure inhibits nuclease activity, which could degrade the ends of chromosomes (7). Another

function of the cap structure is to regulate the activity of telomerase, which is an enzyme responsible for extending the telomere (6).

Telomerase is the solution to the DNA end-replication problem because normal DNA polymerases in the cell cannot replicate the ends of the chromosomes. Due to semiconservative DNA replication and the fact that DNA polymerases can only synthesize DNA in a 5' to 3' direction after the incorporation of a primer, the ends of chromosomes cannot be replicated. Without the action of telomerase, the chromosomes would become gradually shorter after each replication cycle (S phase) resulting in a loss of genetic information (6). Telomerase extends the 3' end of the chromosome by an RNA-templated addition of tandem repeats, which are typically TG-rich sequences (8, 9). Once the 3' terminus is extended by telomerase, the cell can synthesize the complementary strand through lagging strand synthesis, thus, solving the DNA end replication problem without any loss of genetic material (6). In *S. cerevisiae*, telomerase consists of four components which include *TLC1* RNA plus the Est1, Est2, and Est3 proteins (Figure 2) (10-13). The *TLC1* RNA acts as a template upon which the Est2 polymerase can synthesize new DNA. Another protein, Cdc13, is essential for telomerase function *in vivo*. Cdc13 is a telomeric DNA-binding protein, which also interacts with Est1 and aides in the recruitment of telomerase to the telomere (13, 14).

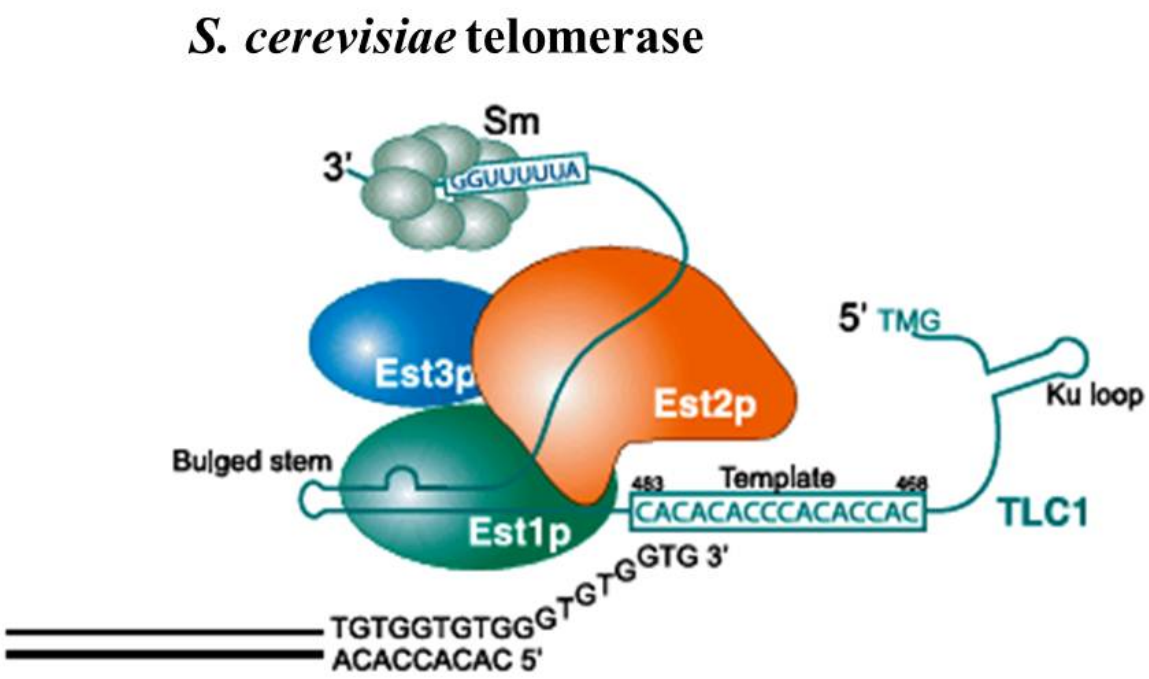

Figure 2. Illustration of the components in telomerase of *S. cerevisiae.* Source: Smogorzewski *et al.* (15).

A major component of the telomere cap structure is the Ku complex (Figure 3). This multifunctional complex is conserved in eukaryotes and in some prokaryotes. In *S. cerevisiae*, the Yku complex is a heterodimer comprised of two subunits which are Yku70 (70 kDa) and Yku80 (80 kDa) (16). The Ku complex was discovered as an autoantigen among patients with autoimmune diseases such as severe combined immunodeficiency (SCID), polymytositis-scleroderma overlap syndrome, and systemic lupus erythematosus (17-20). Thus, it was later discovered that the Ku complex is involved in V(D)J recombination where gene segments are rearranged to form a diverse assortment of immunoglobulin and T-cell receptor genes (20-25). In vertebrates, the Ku80 subunit contains a binding domain for the DNA-dependent protein kinase (DNA-

PK), which is absent in the yeast homolog (26). The Ku complex binds dsDNA ends independent of sequence or structure (blunt, 3' overhang, or 5' overhang) (27-31).

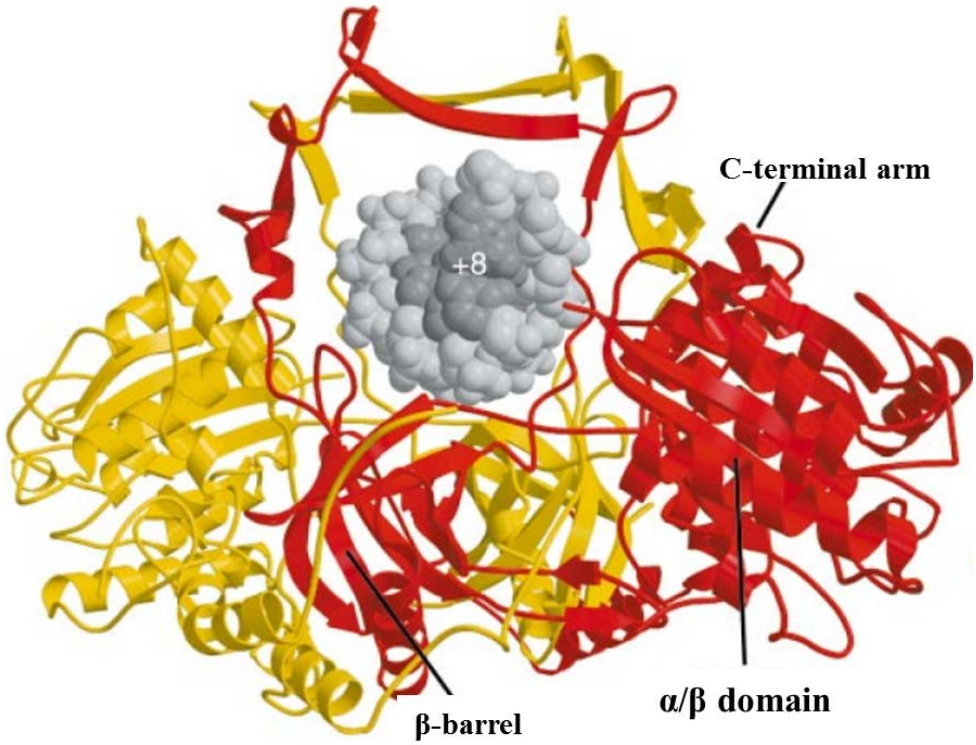

Figure 3. Structure of Ku bound to DNA. Ku70 is shown in red, Ku80 is shown in yellow, and DNA is shown in gray (Modified from reference 32).

Evidence suggests that the Yku complex is a major factor governing the recruitment of telomerase to the telomere through its interaction the *TLC1* RNA component (Figure 4A) (33, 34). Also, the Yku complex prevents deleterious recombination or degradation of the telomere by nucleases such as Exo1 (Figure 4B) (35-40). Another function of the Yku complex is for repressing the transcription of genes near the telomere, which is known as the telomere position effect (TPE) (Figure 4C) (5). This repression is achieved via interactions between the Yku complex, the Rap1 complex,

and the silent information regulator (SIR) complex (Sir2, Sir3, and Sir4). Recent evidence has shown that the Yku80 subunit interacts directly with the Sir4 subunit, which enables the occurrence of TPE (41-44). Also, the Yku complex aids in directing and clustering the telomeres at the nuclear periphery through an interaction with a nuclear porin protein (labeled with an X in Figure 4D) (45-49).

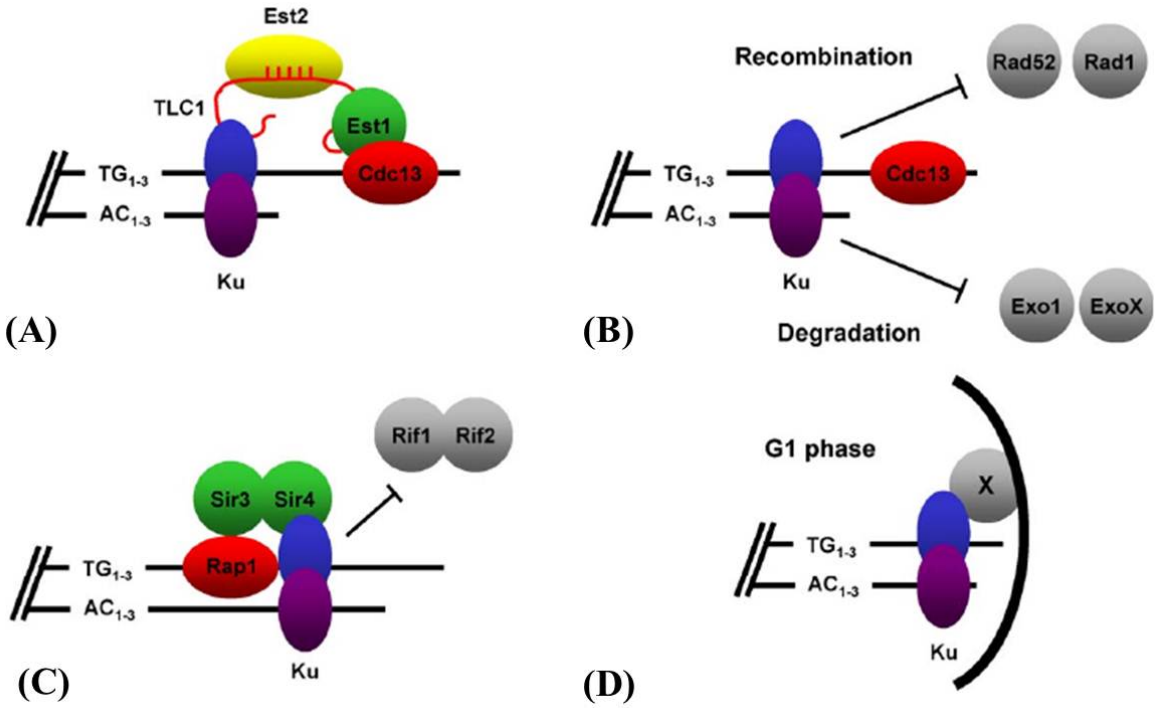

Figure 4. Illustration of the roles the Ku complex participates in at the telomeres of chromosomes in the budding yeast *S. cerevisiae* (Modified from reference 26).

In addition to the various roles at the telomere, the Yku complex is important for the repair of single-stranded and double-stranded DNA lesions resulting from exposure to exogenous DNA damaging agents. DNA DSBs are particularly deleterious to an organism because both strands of DNA are damaged (50). There are multiple causes of

DSBs which include: ionizing radiation, radiomimetic agents, oxidation, enzymatic degradation, physical stress (e.g., chromosomes pulled in opposite directions during mitosis resulting in breakage), and endonucleases (51). There are two known pathways involved in the repair of DSBs. The first pathway, homologous recombination (HR), aligns and rejoins the broken DNA ends using homologous sequences (Figure 5). The first stage of HR involves the processing of DSBs to form single-stranded DNA (ssDNA), which is mediated by the Mrx complex (Mre11, Rad50, and Xrs2) working in conjunction with other enzymes such as Exo1, Sae2, Srs2, and Dna2 (52). The second step in HR involves homologous pairing and strand exchange, which forms a displacement loop (D-loop). This process is mediated by Rad51, Rad52, Rad54, Rad55, and Rad57 along with single-stranded DNA binding protein Rpa (53, 54). The final step in HR is the least understood, but research shows the formation of crossovers via Holliday junctions prior to resolution (55-57). The second pathway, non-homologous end-joining (NHEJ), repairs broken DNA ends independent of sequence homology (Figure 5). The latter pathway is conserved in organisms ranging from bacteria to mammals, but is of particular interest because this is the main DNA repair mechanism in higher eukaryotes (50). Interestingly, several of the proteins required in preserving the stability of telomeres are involved in the NHEJ repair pathway. Comparison of the two pathways shows that the NHEJ pathway is more error-prone, but in higher eukaryotes the genome consists of many non-coding regions, which enables a higher tolerance of mistakes made by NHEJ (58).

The NHEJ repair pathway in *S. cerevisiae* involves at least eight genes which include: *YKU70* (*HDF1*), *YKU80* (*HDF2*), *DNL4* (*LIG4*), *LIF1*, *NEJ1*, *RAD50*, *MRE11*,

and *XRS2*. The proteins encoded by these genes can be sorted among three groups which include: Yku70/Yku80, Dnl4/Lif1/Nej1, and Rad50/Mre11/Xrs2 (58). A fourth complex composed of the Sir2, Sir3, and Sir4 proteins may also play a role, possibly indirectly (51). The exact involvement of these protein complexes is still uncertain, though recent evidence has suggested certain roles for each of the complexes. The Yku70/Yku80 complex binds the ends of DSBs (ssDNA or dsDNA) in a manner which is independent of sequence. The Rad50/Mre11/Xrs2 complex is recruited and then binds to the Yku70/Yku80 complex, thus, forming an end-bridging complex. Mre11 possesses endo- and exonuclease activities that enable the processing of the ends of DSBs prior to ligation. The ligation of the ends is carried out by the Dnl4/Lif1/Nej1 complex resulting in a repaired chromosome (58).

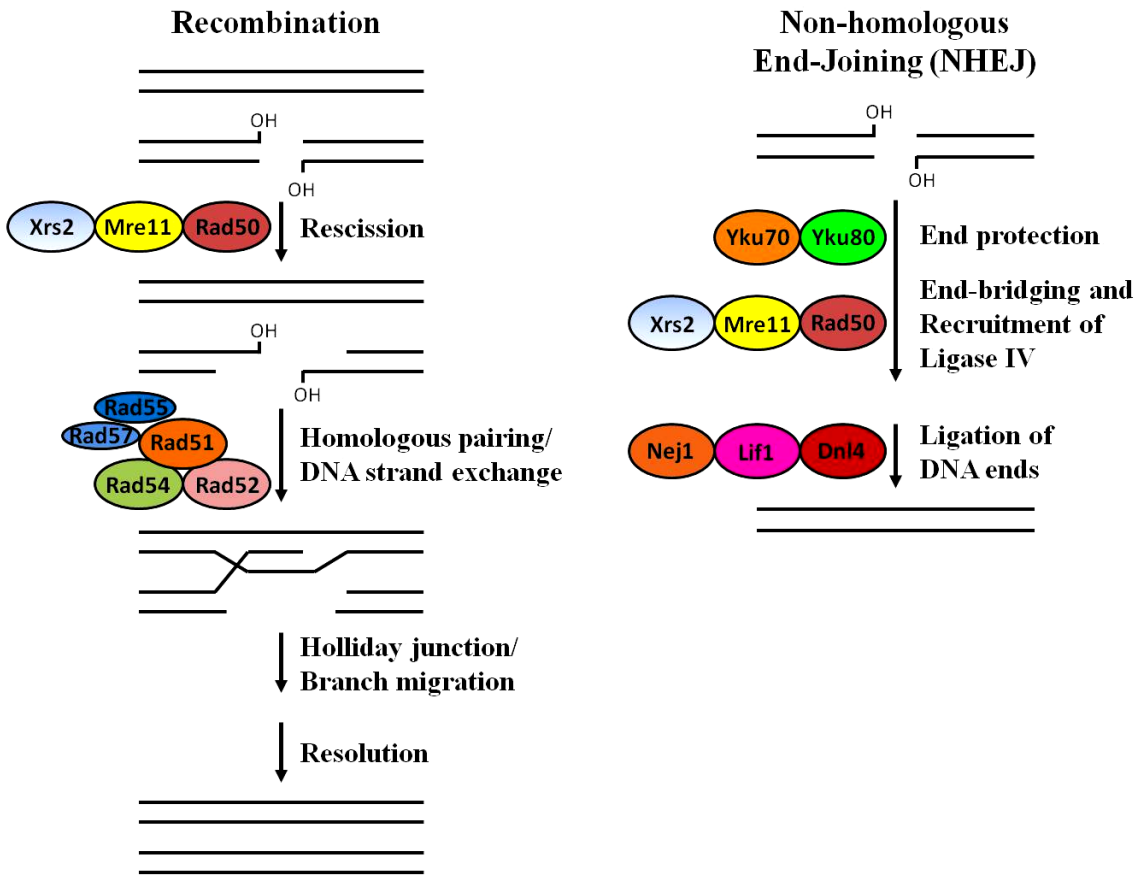

Figure 5. Schematic diagram of the eukaryotic DNA repair pathways of homologous recombination and non-homologous end-joining.

Other proteins involved in telomere maintenance and/or repair of DSBs include Srs2, Sgs1, and Sae2. Both Srs2 and Sgs1 have helicase activity with 3'-5' polarity (59, 60). Yeast cells lacking Sgs1 or Srs2 displayed a shortened life span (59) and *sgs1* mutants were more sensitive to DNA lesions (61). Sae2 is an endonuclease that works with the Mrx complex during DNA repair and is cell cycle regulated (62-64). Pif1 is conserved from yeast to humans and is a helicase that acts as a negative regulator of telomerase (65-67). Evidence has shown an increase in telomere length in *pif1* mutants, which correlates with its telomerase regulatory function (65, 66). Also, overexpression

of Pif1 causes a reduction in telomere-bound Est2 and Est1 (67) and in *pif1 yku70* mutants, temperature sensitivity was suppressed compared to *yku70* mutants (68) indicating that the telomeres were stabilized by removal of the helicase.

Numerous studies have been performed on cells of *S. cerevisiae* lacking Yku70, Yku80, or both. During normal growth at 30 °C, telomeres were observed to be ~200 bp long in the *yku70* mutants compared to ~350 bp in the wildtype. In *yku70* mutants, cell viability was not affected at 30 °C; however, viability was greatly reduced when the mutants were grown at 37 °C. This temperature sensitivity is believed to be caused by instability of the telomeres caused by increased nuclease degradation at the ends and loss of tethering to the nuclear membrane (26). Based on the data and the knowledge of the Yku complex, the *yku70* mutants have reduced ability to efficiently recruit telomerase to the telomeres due to the loss of interaction between the Yku complex and *TLC1* RNA, which resulted in decreased synthesis carried out by the ribonucleoprotein (RNP) complex (69). Further studies of the temperature sensitive phenotype of *yku70* mutants of *S. cerevisiae* revealed that in addition to short telomeres, there were long overhangs of the G-rich strands detected throughout the cell cycle, whereas wildtype cells only display these long overhangs during S phase. Because the Yku complex is a vital component of the telomere end cap structure, cells deficient in the Yku70 subunit have unstable telomere end cap structures. This instability gives access to nucleases such as Exo1, which degrades DNA ends in a 5' to 3' direction. The observation that *yku70* mutants are viable at 30 °C, but arrest growth in G_2 phase at 37 °C, suggests that the cell cycle is arrested at 37 °C because the cells activate a DNA damage checkpoint due to the long overhangs of the G-rich strand (70). Another phenotype observed in *yku* mutants of *S.*

cerevisiae is that TPE is defective. The data suggests that this phenotype is due to the fact that the Sir4 protein has lost interactions with Yku80, which facilitates the recruitment of the Sir3 protein. The loss of these interactions resulted in the diminished capability of the cell to silence transcription of telomere-proximal genes (44). Another phenotype observed in *yku70* mutants is the inability of the cell to tether the telomeres at the nuclear periphery (71). Finally, *yku* mutants show severely decreased efficiency in the NHEJ repair pathway (72).

As described above, *yku* mutants of *S. cerevisiae* exhibit decreased chromosome stability at 37 °C, whereas wildtype cells undergo a heat-shock response but continue growing. Research has shown that overexpression of the telomerase components *EST2* (the catalytic subunit) and *TLC1* (the RNA subunit) can rescue the temperature sensitivity of *yku70* or *yku80* mutants (73-77). The mechanism of rescue is unclear, but is likely to involve protection of the DNA ends from nucleases. The effectiveness of other telomerase proteins such as Est1, Est3 and Cdc13 in rescue of *yku* mutants is not yet clear.

The goal of the current project was to further characterize the involvement and roles of the Yku complex in maintaining chromosome stability in cells of *S. cerevisiae*. The specific functions of the Yku complex and the precise reasons for the observed phenotypes of *yku* mutants are still not understood. The rescue of chromosome instability in *yku* mutants of *S. cerevisiae* at 37 °C by cloning and overexpressing the telomerase genes *EST1* and *EST3* was analyzed and compared to the effects of telomerase genes *TLC1* and *EST2*. Also, analysis of the potential of thirteen other genes from a cDNA library to suppress chromosome instability in *yku* mutants of was performed.

Comparisons were made between the abilities of overexpressing these genes to that of overexpressing telomerase subunits. Also, analysis of temperature sensitivity was performed on *yku70 sae2*, *yku70 srs2*, and *yku70 pif1* double mutants at 37 °C and the ability of *TLC1* RNA and Est2 polymerase overexpression to rescue *yku70 sae2*, *yku70 srs2*, and *yku70 pif1* double mutants at 37 °C was examined. Furthermore, during the course of this work modifications to existing methodologies were developed to improve the quality of chromosomal DNA purified from wildtype and mutant yeast cells.

CHAPTER II

MATERIALS AND METHODS

I. MATERIALS

General reagents

Agarose was purchased from EMD Chemicals, Inc. (Gibbstown, NJ). Ethidium bromide was purchased from IBI Scientific (Peosta, IA). TAE running buffer (50X) was purchased from Omega Bio-Tek (Norcross, GA). Lithium acetate dihydrate, polyethylene glycol (PEG-4000), potassium acetate, and calcium chloride dehydrate were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Sodium chloride was purchased from Fisher Scientific (Fair Lawn, NJ). Sodium acetate anhydrous and sodium dodecyl sulfate were purchased from Mallinckrodt Baker, Inc. (Paris, KY).

Enzymes and PCR reagents

Restriction enzymes, Antarctic phosphatase, RNase A, RNase I_f, and Phusion® high-fidelity DNA polymerase were purchased from New England Biolabs, Inc. (Ipswich, MA). RNase V1 biochemistry grade and RNase Cocktail™ (a mixture of RNase A and RNase T1) were purchased from Invitrogen Life Technologies (Grand Island, NY).

Bacteriological and yeast media

Ampicillin (Amp) and all amino acids were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). SOC broth media was purchased from TEKnova, Inc. (Hollister, CA). D-(+)-glucose (anhydrous) was purchased from Mallinckrodt Baker, Inc. (Paris, KY). Bacto agar, bacto yeast extract, bacto peptone, bacto tryptone, and yeast nitrogen base dropout media were purchased from Becton, Dickinson and Company (Sparks, MD).

Cell culture solutions and media

Non-selective YPDA yeast plate growth media was comprised of 0.001% adenine, 1% bacto yeast extract, 2% bacto agar, 2% glucose, and 2% bacto peptone. YPDA liquid media was prepared in with the same concentrations as YPDA, but minus agar. Synthetic media containing drop-out mix (all essential amino acids minus leucine or uracil, 2% bacto agar, 2% glucose, 0.5% ammonium sulfate, 0.17% yeast nitrogen base without ammonium sulfate or amino acids) was used for plasmid selection of growing yeast cells.

LB + Amp plates (0.01% Amp, 0.5% NaCl, 0.5% yeast extract, 1% bacto tryptone, 1.5% agar) or LB + Amp broth (as plates, but without agar) were used to grow *E. coli* cells.

Strains and plasmids

All yeast strains and plasmid DNAs used in this study are listed in Tables 1 and 2.

Table 1. Strains used in this project.

Strain	Genotype	Source
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	74
As BY4742, but	*sgs1Δ::G418^r*	Lab strain
As BY4742, but	*sae2Δ::G418^r*	Lab strain
As BY4742, but	*srs2Δ::G418^r*	Lab strain
As BY4742, but	*pif1Δ::G418^r*	Lab strain
YLKL652	BY4742, *yku70Δ::HIS3*	Lab strain
YLKL857	BY4742, *yku70Δ::G418^r*	Lab strain
YLKL1184	*sae2Δ::G418^r yku70Δ::URA3*	This study
YLKL1185	*srs2Δ::G418^r yku70Δ::URA3*	This study
YLKL1186	*pif1Δ::G418^r yku70Δ::URA3*	This study

Table 2. Plasmids employed for this project.

Plasmid	Description	Source
pRS315	*CEN/ARS LEU2*	79
pRS316	*CEN/ARS URA3*	79
p426TEF	*2μ URA3*	80
pGEM4Z S-H/URA	*yku70Δ::URA3*	81
pVL715	*2μ ADH1p::EST2 URA3*	82
pVL999	*2μ ADH1p::EST2 LEU2*	82
pLKL64Y	*2μ ADH1p::TLC1 LEU2*	Lab plasmid
pLKL83Y	*2μ ADH1p::TLC1 URA3*	Lab plasmid
pLKL92Y	*p426TEF + EST1*	This work
pLKL93Y	*p426TEF + EST3*	This work
pKu-S16	*yku70* 37 °C sensitive suppressor: *TLC1*	77
pKu-S28	*yku70* 37 °C sensitive suppressor: *HYP2*	77

II. METHODS

Gel electrophoresis

Gel electrophoresis was performed using 0.6-1.5% agarose gels in 1X TAE running buffer in a Life Technologies Horizon 11-14 gel rig. A Kodak IS440 CF imaging system with Kodak 1D imaging software was used to image agarose gels stained with ethidium bromide.

DNA transformations

Transformations of plasmids into yeast strains were performed using the rapid lithium acetate/DMSO method described by Soni *et al*. (83). DNA transformations for gene disruption were performed using the high efficiency lithium acetate method described by Gietz *et al*. (84). *E. coli* transformations were performed using the Chung and Miller method (85) or as recommended by New England Biolabs for *E. coli* 5-alpha cells.

Plasmid DNA purification

Plasmid DNA was purified using the QIAprep® Spin Miniprep Kit and protocol provided by QIAGEN, Inc. (Valencia, CA).

Polymerase Chain Reaction

An Applied Biosystems 2720 Thermal Cycler was used for PCR reactions, which consisted of 30-40 cycles. The individual cycles involved a denaturation temperature of

94 °C for 30 seconds, annealing temperatures from 50-54 °C for 40 seconds, and an extension temperature of 72 °C for 60-120 seconds.

Dilution pronging cell survival assays

Cells were harvested into YPDA broth, incubated at 37 °C for 2 hours, pelleted and resuspended in sterile ddH_2O, and diluted 1/50. The cell solution was sonicated for 8 seconds at 2 watts using a Sonics Vibracell Ultrasonic Processor (Newtown, CT) followed by quantitation of the cells on a Reichert (Buffalo, NY) hemocytometer using a trinocular compound microscope (Model M837T). At a concentration of 1 x 10^7 cells per 220 µl, yeast cells were added to a microtiter dish followed by 5-fold serial dilutions 5 times across the length of the dish. Glucose minus uracil or leucine plates were then used to prong the cells. Pronged cells used as positive cell viability controls were incubated at 30 °C and cells used for experimentation were incubated at 37 °C. Cells were grown for 3-4 days and a Canon Powershot G3 digital camera was used to image the plates. All images were saved as JPEG files.

Yeast strain construction

yku70Δ::URA3 double mutant constructs

HindIII + EcoRI-digested *YKU70* deletion plasmid pGEM4Z S-H/URA was transformed into single mutant cells to create yeast strains YLKL1184 (*sae2Δ::G418*^{r} *yku70Δ::URA3*), YLKL1185 (*srs2Δ::G418*^{r} *yku70Δ::URA3*), and YLKL1186 (*pif1Δ::G418*^{r} *yku70Δ::URA3*). Following transformation, cells were spread on glucose minus uracil plates. Then, fresh glucose minus uracil plates were used to patch resulting

colonies. The resulting patches were replica-plated and double-imprinted to new glucose minus uracil plates, followed by incubation at either 37 °C or 39 °C for three days. Extended analysis was performed on cells that displayed the temperature sensitivity phenotype. However, YLKL1186 (*pif1Δ::G418*^{r} *yku70Δ::URA3*) patches were further analyzed without the temperature sensitivity phenotype. The *yku70* disruption of the resulting double mutants was confirmed by PCR using the primers Ku70a (TTGAGATC GGGCGTTCGACTCGCC) and Ku70b (TGCTAATAGTTGTACAGTACAA CGT), followed by confirmation via gel electrophoresis.

TLC1 RNA, Est2 polymerase, and Hyp2 rescue of the temperature sensitivity of yku70 cells

The rescue of *TLC1* RNA, Est2 polymerase, and Hyp2 overexpression on *yku70* temperature sensitivity was assayed by replica plating and pronging. YLKL857 (BY4742, *yku70Δ::G418*^{r}) and YLKL652 (BY4742, *yku70Δ*::*HIS3*) were independently transformed with pRS316 (*CEN/ARS URA3*), pVL715 (*2μ ADH1p::EST2 URA3*), pKu-S16 (*yku70* 37 °C sensitive suppressor: *TLC1*), and pKu-S28 (*yku70* 37 °C sensitive suppressor: *HYP2*). Cells were spread on glucose minus uracil plates and grown at 30 °C for 3 days. Resulting colonies were patched on fresh glucose minus uracil plates and grown at 30 °C for 2 days. Three isolates from each transformant were replica-plated to glucose minus uracil plates and grown at 30 and 37 °C for 3 days to assess the rescue of the temperature sensitivity phenotype of *yku70* cells. Pronging tests were performed essentially as described above.

Cloning of EST1 and EST3 and rescue of the temperature sensitivity of yku70 cells

The rescue of *yku70* temperature sensitivity was assayed under conditions resulting in overexpression of *EST1* and *EST3*. The 100 µl plasmid digest reaction for *EST3* cloning was comprised of 8 µl p426TEF DNA, 1X BSA, 1X NEB buffer 2, 70 U XhoI, and 35 U SpeI. The 100 µl plasmid digest reaction for *EST1* cloning was comprised of 8 µl p426TEF DNA, 1X BSA, 1X NEB buffer 2, and 40 U SpeI. Both digests were incubated at 37 °C for 2 hours. Aliquots were ethanol precipitated, followed by confirmation via gel electrophoresis. For *EST3* cloning, the digested p426TEF was phosphatased with the 40 µl reaction mixture comprised of 1,500 ng SpeI-cut p426TEF, 1X Antarctic phosphatase buffer, and 5 U Antarctic phosphatase. The reaction mixture was incubated at 37 °C for 15 minutes, followed by heat inactivation at 65 °C for 5 minutes. NaOAc was added to a final concentration of 0.3 M, followed by the addition of 2.5 volumes of 100% ethanol. The resulting solution was centrifuged at 16,000 x g for 10 minutes and washed with 200 µl 70% ethanol. The pellet was dried and resuspended in 12 µl ddH_2O. For both plasmid digestions, the DNA concentrations were determined by fluorometry.

EST3 was amplified by PCR and the 50 µl reaction mixtures were comprised of 2 µl BY4742 chromosomal DNA, 1 µM Est3A primer (GACTCAAACTAGTAAGCTTG TAAACAATGCCGAAAG), 1 µM Est3B primer (GACTACAACTAGTAAGCTTGTT TCTCTAGAGGAGTA), 0.25 mM dNTPs, 1X Phusion buffer, 1 mM Mg^{2+}, and 2 U Phusion polymerase. *EST1* was amplified by PCR with the same reaction mixture except with no Mg^{2+} and with different primers, which are Est1A primer (GACTCAAACTAGT AAGCTTGATAATGGATAATGAAG) and Est1B primer (GACTCAACTCGAGAAG

CTTTACTTGTTCTCTCAAGT). Both *EST1* and *EST3* PCR products were confirmed via gel electrophoresis. Amplified *EST3* DNA was digested in a 100 µl reaction mixture comprised of 10 µl *EST3* gene fragment, 1X BSA, 1X NEB buffer 2, and 40 U SpeI. Amplified *EST1* DNA was digested in a 100 µl reaction mixture comprised of 10 µl *EST1* gene fragment, 1X BSA, 1X NEB buffer 2, 70 U Xho1, and 35 U SpeI. Both *EST1* and *EST3* DNA digests were incubated at 37 °C for 2 hours. Aliquots were ethanol precipitated and confirmed by gel electrophoresis, followed by concentration determination via fluorometry.

The *EST1* + p426TEF ligation mixture (15 µl) was comprised of 150 ng digested *EST1* DNA, 80 ng digested p426TEF DNA (SpeI/XhoI), 1X T4 ligase buffer, and 480 U T4 DNA ligase. The *EST3* + p426TEF ligation mixture (15 µl) was comprised of 200 ng digested *EST3* DNA, 200 ng digested p426TEF DNA (SpeI), 1X T4 ligase buffer, and 480 U T4 DNA ligase. Both ligations were incubated at room temperature for 3.5 hours, ethanol precipitated, resuspended in H_2O, and 7.5 µl of each ligation reaction mixture were transformed into NEB 5-alpha High Efficiency competent *E. coli* cells. Plasmid preps were performed, followed by SacI/EcoRI digests (pEST3) and SpeI/XhoI digests (pEST1) and gel electrophoresis for confirmation. Orientation was confirmed with SacI/NdeI digests (pEST1) and SacI/MscI digests (pEST3), followed by gel electrophoresis (See results).

EST1 expression plasmid pLKL92Y (p426TEF + *EST1*) and *EST3* expression plasmid pLKL93Y (p426TEF + *EST3*) were independently transformed into YLKL857 (*yku70Δ::G418*^{r}) cells and spread to glucose minus uracil plates. *TLC1* RNA expression plasmid pLKL83Y (*2µ ADH1p::TLC1 URA3*) and pRS316 (*CEN/ARS URA3*) were

independently transformed into YLKL857 (*yku70Δ::G418*^{r}) and spread to glucose minus uracil plates to be used as positive and negative controls, respectively. After 2 days of growth at 30 °C, resulting colonies were then patched to fresh glucose minus uracil plates and grown at 30 °C for 2 days. Cells were then harvested, counted, and pronged on glucose minus uracil plates to determine the rescue effect of supracellular levels of Est1 and Est3 proteins on *yku70* cell temperature sensitivity. Cells were allowed to grow for 3 days at 30 °C and 37 °C prior to analysis.

Sequencing of *EST1* and *EST3* was performed by Retrogen, Inc. using two independent isolates of both pLKL92Y (p426TEF + *EST1*) and pLKL93Y (p426TEF + *EST3*) and the primers T7 (TAATACGACTCACTATAGGG), Tef1a1 (CTTTCGATGA CCTCCCATTGATATT), Est1-583 (GTGATTAATCT AGGTTCCACACAT), and Est1-1181 (CAGATTTGAGTGAGCGCCAGGTTT). The resulting sequences obtained from pLKL92Y and pLKL93Y were then compared to their published sequences in the Saccharomyces Genome Database (www.yeastgenome.org) using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov).

Effect of disruptions of SRS2, SAE2, and PIF1 on temperature sensitivity of yku70 cells

To assess the effect of inactivation of *SRS2*, *SAE2*, and *PIF1* on *yku70* temperature sensitivity, the double mutants YLKL1184 (*sae2Δ::G418*^{r} *yku70Δ::URA3*), YLKL1185 (*srs2Δ::G418*^{r} *yku70Δ::URA3*), and YLKL1186 (*pif1Δ::G418*^{r} *yku70Δ::URA3*) were constructed as previously described. Two isolates from each double mutant and YLKL652 (BY4742, *yku70Δ::HIS3*) transformed with pRS316

(*CEN/ARS URA3*) were patched to fresh glucose minus uracil plates and grown at 30 °C for 2 days. The cells were then incubated at 37 °C for 2 hours in YPDA broth, harvested, counted, and pronged to new glucose minus uracil plates and grown at 30 and 37 °C for 3 days to assess the effect on *yku70* temperature sensitivity.

TLC1 RNA and Est2 polymerase rescue of yku70, yku70 sae2, and yku70 srs2

The effect of *TLC1* RNA and Est2 polymerase overexpression on *yku70, yku70 sae2, and yku70 srs2* temperature sensitivity was assayed. YLKL652 (BY4742, *yku70::HIS3*), YLKL1184 (*sae2Δ::G418^r yku70Δ::URA3*), and YLKL1185 (*srs2Δ::G418^r yku70Δ::URA3*) cells were each transformed independently with pRS315 (*CEN/ARS LEU2*), pVL999 (*2μ LEU2 ADH1p::EST2*), and pLKL64Y (*2μ LEU2 ADH1p::TLC1*). Cells were spread onto glucose minus leucine plates and grown at 30 °C for 3 days. Resulting colonies were patched on fresh glucose minus leucine plates and grown at 30 °C for 2 days. Two isolates from each transformation were then incubated for 2 hours in YPDA broth, harvested, counted, and pronged to glucose minus leucine plates and grown at 30 and 37 °C for 3 days to assess the rescue of the temperature sensitivity phenotype.

Removal of RNA from yeast chromosomal DNA preps

Optimization of RNase conditions

BY4742 chromosomal DNA preps prepared using the SDS lysis method of Lee *et al.* (86) were initially incubated at room temperature for 5 minutes, followed by the addition of RNase A to a final concentration of 0.4 μg/μl. The DNA/RNA solutions

(~500-1000 ng/µl) were then incubated at 37 °C for 30 minutes and aliquots were subjected to gel electrophoresis on a 1.4% agarose gel. Gels for these experiments were run at relatively high voltage (typically 160 volts) using short run times (~15 minutes) to minimize RNA diffusion. Similar assays were performed using RNase I_f.

A series of reactions were performed on BY4742 chromosomal DNA preps with contaminating RNA included: the addition of RNase Cocktail (24 U/ml RNase A and 1.0 U/µl RNase T1 final concentrations); addition of RNase V1 (5 U/ml final concentration) without buffer; addition of RNase V1 (5 U/ml final concentration) with RNA structure buffer (1X final concentration); addition of RNase Cocktail (24 U/ml RNase A and 1.0 U/µl RNase T1 final concentrations) and RNase V1 (5 U/ml final concentration); and addition of RNase V1 (5 U/ml final concentration), RNase Cocktail (24 U/ml RNase A and 1.0 U/µl RNase T1 final concentrations), and RNA structure buffer (1X final concentration). Resulting reaction mixtures were incubated at 37 °C for 30 minutes and aliquots were subjected to gel electrophoresis on a 1.0% agarose gel.

In some experiments, BY4742 chromosomal DNA was incubated at 65 °C for 5 minutes to denature or destabilize the RNA, enabling different types of RNases to digest the RNA more efficiently. Following this incubation, RNase Cocktail (RNase A and RNase T1) was added to final concentrations of 80 U/ml and 3.3 U/µl, respectively. Also, RNase V1 was added to a final concentration of 5 U/ml. The solutions were then incubated at 37 °C for 30 minutes and aliquots were subjected to gel electrophoresis on a 1.0% agarose gel.

Purification of chromosomal DNA from RNA using columns

Initial attempts to purify BY4742 chromosomal DNA from RNA utilized the QIAprep® Spin Miniprep Kit and the protocol provided by QIAGEN, Inc. (Valencia, CA). Also, CHROMA SPIN™-1000 columns and the protocol provided by Takara Bio Co. (Mountain View, CA) were used to purify BY4742 chromosomal DNA. Resulting DNA solutions were subjected to gel electrophoresis on 1.0% agarose gels.

Purification of chromosomal DNA from RNA using alcohol precipitation

The RNA/protein removal method described by Feliciello and Chinali was also used to purify BY4742 chromosomal DNA from RNA (87). The standard published protocol involved the addition of 1.2 volumes of an 88% isopropanol/0.2 M KOAc mixture to a DNA solution. The resulting mixture was then incubated at room temperature for 10 minutes, followed by centrifugation at 16,000 x g for 5 minutes. The supernatant was removed and the DNA pellet was centrifuged again for 30 seconds, followed by the removal of the remaining supernatant. The DNA pellet was dried and resuspended in the same volume as the original DNA solution in sterile ddH_2O. The different parameters of this protocol varied in order to improve it included: centrifugation for 1, 2, and 5 minutes; use of final concentrations of KOAc of 0.03, 0.05, 0.08, and 0.11 M; and the addition of 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, and 1.2 volumes of an 88% isopropanol/0.1 M KOAc mixture. Resulting DNA solutions were subjected to gel electrophoresis on 1.0% agarose gels.

CHAPTER III

RESULTS AND DISCUSSION

In eukaryotic cells, inactivation of the Ku complex results in many phenotypes that are deleterious to the organism. Upon deletion of Ku in humans, cells are unable to utilize the NHEJ DNA repair pathway, and therefore the immune system is deficient in V(D)J recombination. In yeast, there are numerous phenotypes that appear in *yku70* mutants, including shorter, but stable telomeres at the normal growth temperature of 30 °C and decreased cell viability when mutants are grown at the elevated temperature of 37 °C. The temperature sensitivity observed at this elevated temperature results from telomere instability caused by increased nuclease degradation and the inability to tether the telomeres at the nuclear periphery. At this elevated temperature, the telomere cap becomes further destabilized, enabling nucleases more access, which results in long overhangs of the G-rich strands that persist throughout the cell cycle. Also, these mutant cells have reduced ability to recruit telomerase to the telomeres, which is caused from the loss of interaction between the Yku complex and *TLC1* RNA. Previous research has shown that overexpression of *TLC1* RNA or Est2 protein can rescue the temperature sensitivity of *yku* mutants; however, the precise mechanism involved is still unclear (73-77). This thesis utilized *S. cerevisiae* cells to further investigate the overexpression of telomerase subunits in *yku70* mutants at elevated temperatures.

This project attempted initially to follow up the work of a previous student, Cory Holland, who identified 14 genes that rescued the killing of *yku70* mutants at 37 °C when

they were overexpressed (77). In the earlier work, the mutant cells were transformed with a cDNA library in which each plasmid contained a different yeast gene under the control of a strong *GAL1* promoter (88). Plasmids were identified that permitted growth of *yku70* cells at 37 °C. A total of 25 plasmids were isolated out of ~70,000 that were tested and these corresponded to 14 different genes because some of the genes were obtained multiple times. One of the genes isolated was *TLC1*, which had been previously shown to rescue the death of *ku* mutants at elevated temperatures. Several of the other genes had previously been associated with protein synthesis (*ANB1*, *HYP2*, *RRP14*, *BUD21*, *RPL9A*, and *HUG1*). Other genes affected nucleic acid metabolism (*URA5*, *RTF1*, *REP1*, and *MPP6*) or other processes (*CPR1* and *YLR003C*).

A method commonly employed to study the temperature sensitivity of *yku* mutants is dilution pronging assays (Figure 6). In this semi-quantitative assay, cells lacking desired gene(s) and/or expressing desired gene(s) are separately added in equivalent amounts into the first column of a 96-well microtiter dish, followed by 5-fold dilutions across the next 5 rows. A pronger is placed in the microtiter dish and the cells are transferred to a Petri dish containing a desired medium. The cells are then incubated at various temperatures depending on the experiment being performed. After incubation, the level of cell death or cell rescue can be quantitatively measured.

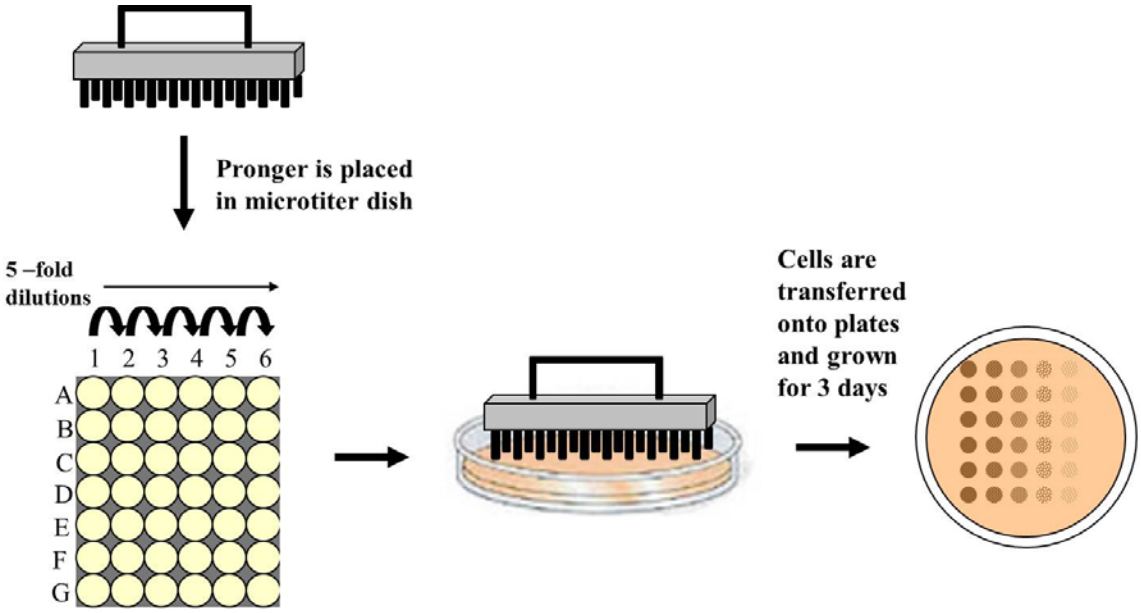

Figure 6. Schematic illustration of a dilution pronging assay to quantitate cell survival.

In preliminary studies for these experiments, the abilities of *TLC1* RNA and *EST2* to rescue *yku70* cells were tested using strains YLKL652 (*MATα yku70::HIS3*) and YLKL857 (*MATα yku70::G418*^{r}) to determine which strain produced the best results. The plasmids pRS316 (*URA3*), pLKL83Y (*ADH1p::TLC1 URA3*), and pVL715 (*ADH1p::EST2 URA3*) were independently transformed into YLKL652 and YLKL857. Transformants were subsequently pronged to glucose minus uracil plates and grown at 30 °C and 37 °C for 3 days. As shown in Figure 7, overexpression of both genes resulted in strong growth of *yku70* cells at 37 °C compared to the pRS316 vector control in both strains.

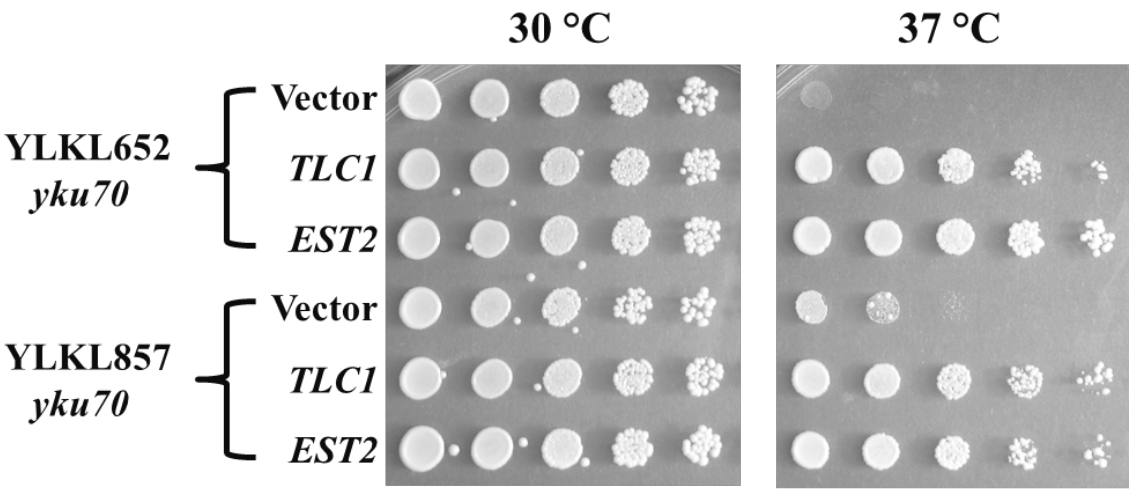

Figure 7. Rescue of *yku70* mutants at 37 °C by overexpression of *EST2* and *TLC1* RNA. Vector, pRS316 control plasmid; *TLC1*, pLKL83Y (*ADH1p::TLC1*); *EST2*, (*ADH1p::EST2*).

Next, several attempts were made to reproduce the results of C. Holland using YLKL652 and YLKL857. Unfortunately, tests using 6 of the new genes did not show any rescue either by means of simple replica-plating or dilution pronging assays as shown for Figure 7. Attempts were made to use other *yku70* mutants such as YLKL843 (*MATα yku70::HIS3*), but none of the cDNA overexpression plasmids could alleviate the growth defect of the cells. Variation of temperature and plate media did not improve these results. It was therefore decided to drop this portion of the project.

Past work in the Lewis lab and elsewhere has demonstrated that overexpression of the telomerase subunits *EST2* and *TLC1* can rescue *ku* cells, but not overexpression of Cdc13 (73-77). Overexpression of the Est3 subunit has not been tested and results with *EST1* have been contradictory. A previous report by Nugent *et al.* (73) indicated that Est1 could rescue, but these results could not be reproduced in the Lewis lab using the same plasmid (results not shown). To resolve this discrepancy and expand this work,

EST1 and *EST3* were cloned and overexpressed from a strong, constitutive yeast promoter. p426TEF was chosen for the next set of experiments as a cloning vector for the telomerase genes *EST1* and *EST3* (Figure 8). This vector contains a strong, constitutive *TEF1* promoter, *CYC1* terminator, *URA3* selectable marker, and numerous restriction sites including XhoI, SpeI, KpnI, and SacI.

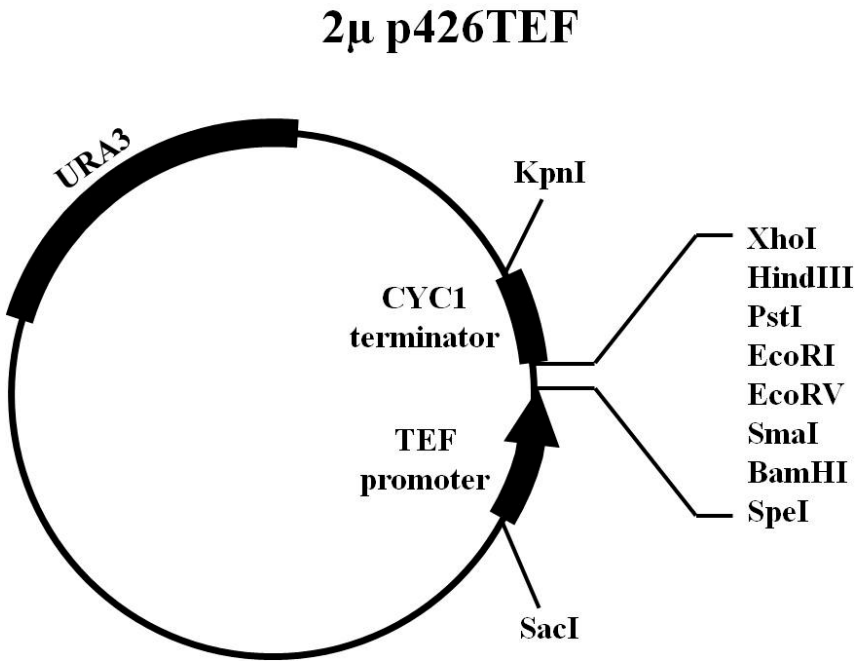

Figure 8. Diagram of the cloning vector p426TEF used to create p426TEF::EST1 and p426TEF::EST3.

The overall procedure for cloning each gene into p426TEF is shown in Figure 9. First, forward and reverse primers containing specific restriction sites were designed for the desired gene, followed by PCR amplification. Next, both the gene fragment and vector were digested with the same restriction enzymes. Finally, the digested vector and

gene fragment were ligated together with T4 DNA Ligase, forming a plasmid encoding a specific gene of choice.

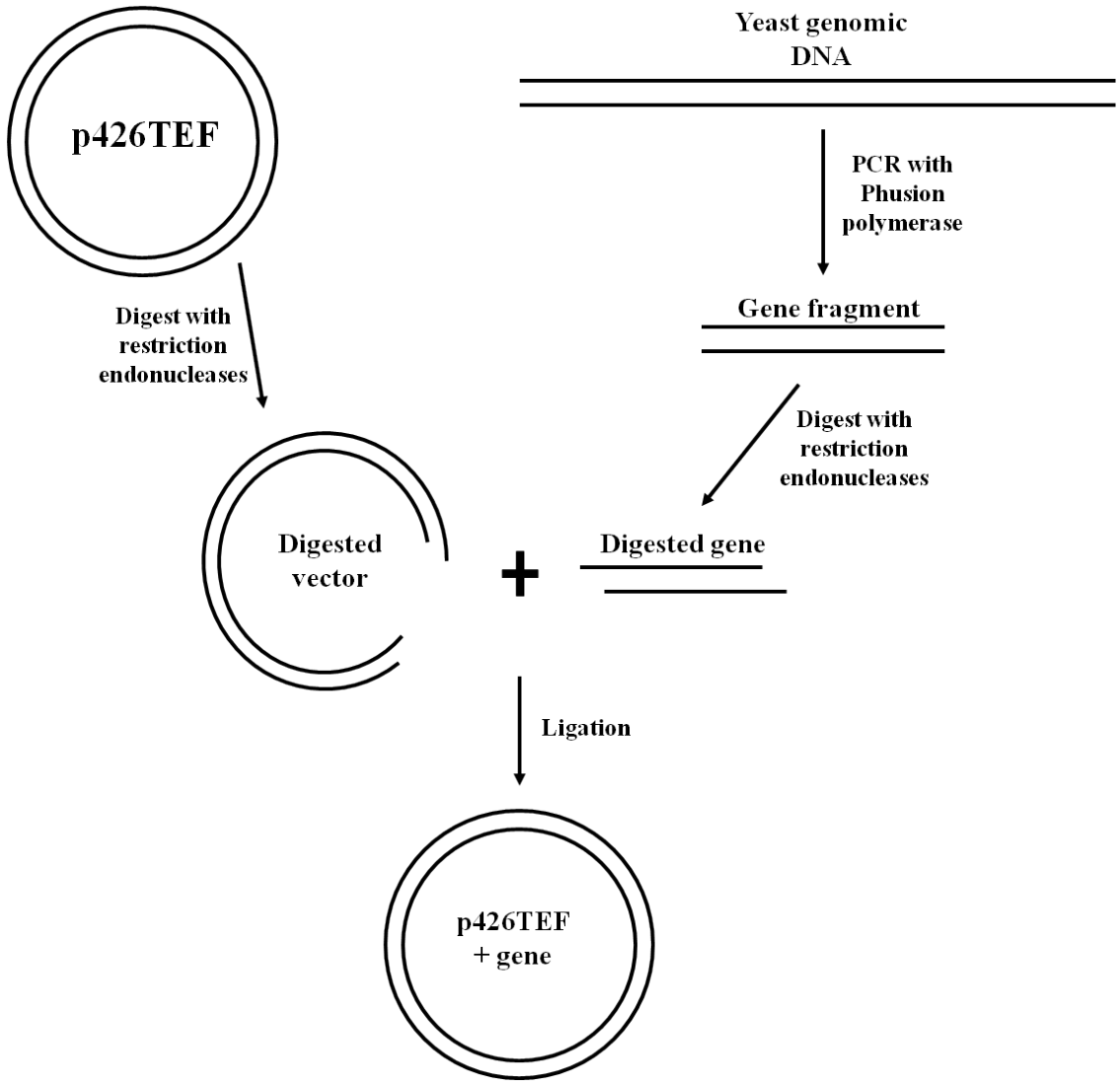

Figure 9. Schematic illustration of the cloning of the *EST1* and *EST3* genes into p426TEF.

The *EST1* gene (2100 bp) was PCR amplified with primers Est1A and Est1B, followed by confirmation of the PCR product (2160 bp with flanks) on an agarose gel (Figure 10A). The *EST1* gene PCR fragment and p426TEF vector were digested with

SpeI and XhoI, purified, and mixed for ligation. The ligation mix was transformed into 5-alpha competent *E. coli* cells and spread onto LB + Amp plates. DNA minipreps were performed from individual colonies and run alongside vector controls. An example of 8 such uncut plasmids is shown in Figure 10B. Out of 20 plasmids tested, 6 appeared to have inserts.

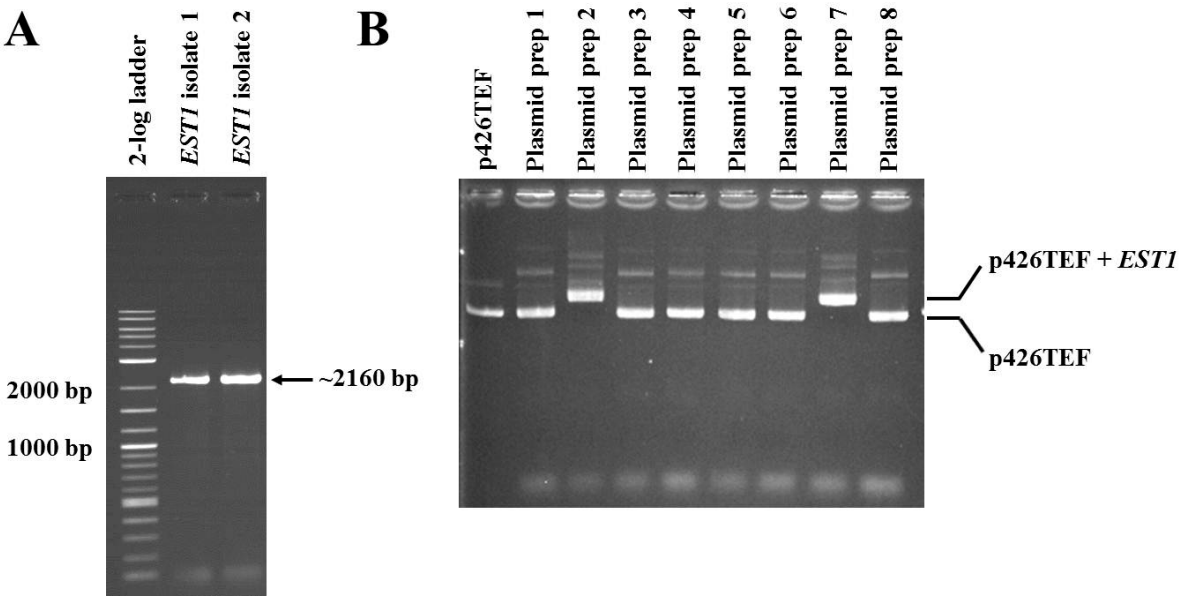

Figure 10. A. A 0.9% agarose gel containing PCR amplified *EST1* gene fragment. B. A 0.7% agarose gel screening for p426TEF::EST1 plasmids purified from *E. coli.*

In order to determine whether the gene was inserted into the p426TEF vector, p426TEF::EST1 plasmids with inserts were digested with SpeI and XhoI, followed by gel electrophoresis on a 1.2% agarose gel (Figure 11). Lane 2 is the SpeI and XhoI digested vector p426TEF without any gene insert. The band at ~2100 bp in lanes 3, 4, 5, and 6 represents the *EST1* gene insert from 4 independent preps. This gel confirms that an

insert of the correct size was cloned and that the original restriction sites flanking the gene were not altered during the cloning.

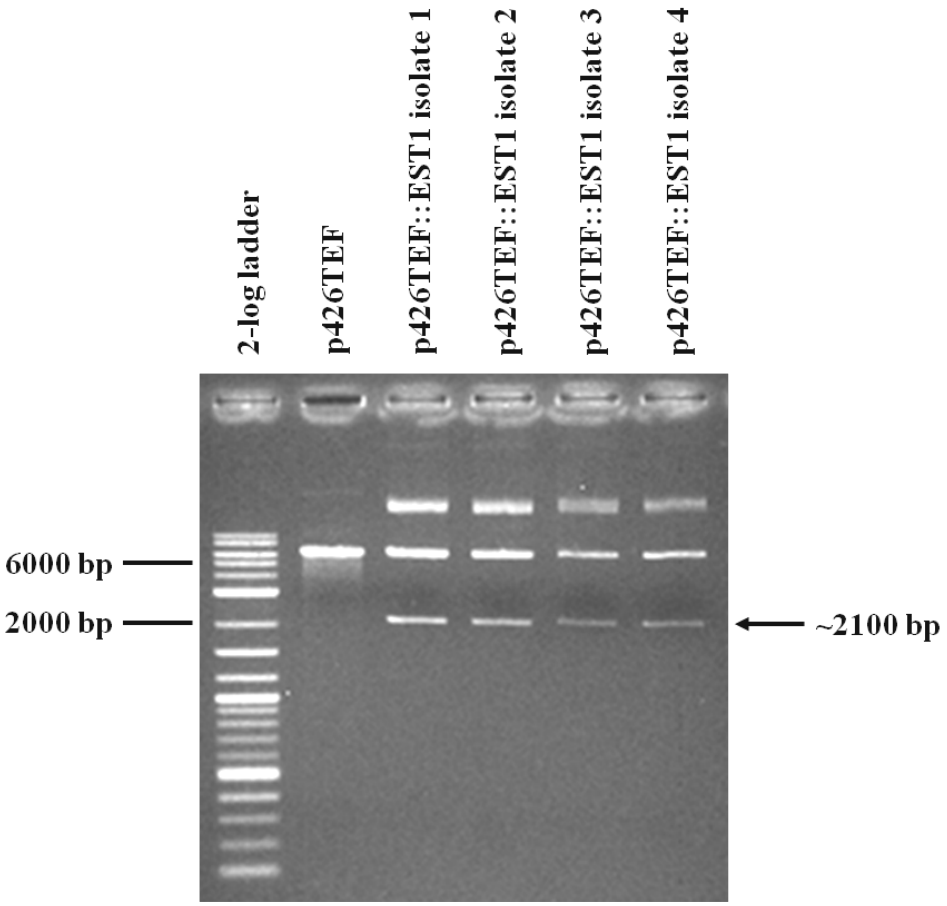

Figure 11. A 1.2% agarose gel of restriction digestions of p426TEF::EST1 plasmids with SpeI and XhoI demonstrating the presence of *EST1*.

In order to determine the orientation of the *EST1* gene inserted into the cloning vector p426TEF, a restriction digest with SacI and NdeI was performed. Figure 12A illustrates an example of digestion of an *EST1* plasmid with correct orientation resulting in a 2102 bp DNA fragment and a 2341 bp DNA fragment. Figure 12B illustrates an

example of digestion of a plasmid with the opposite orientation resulting in an 816 bp DNA fragment and a 3627 bp DNA fragment.

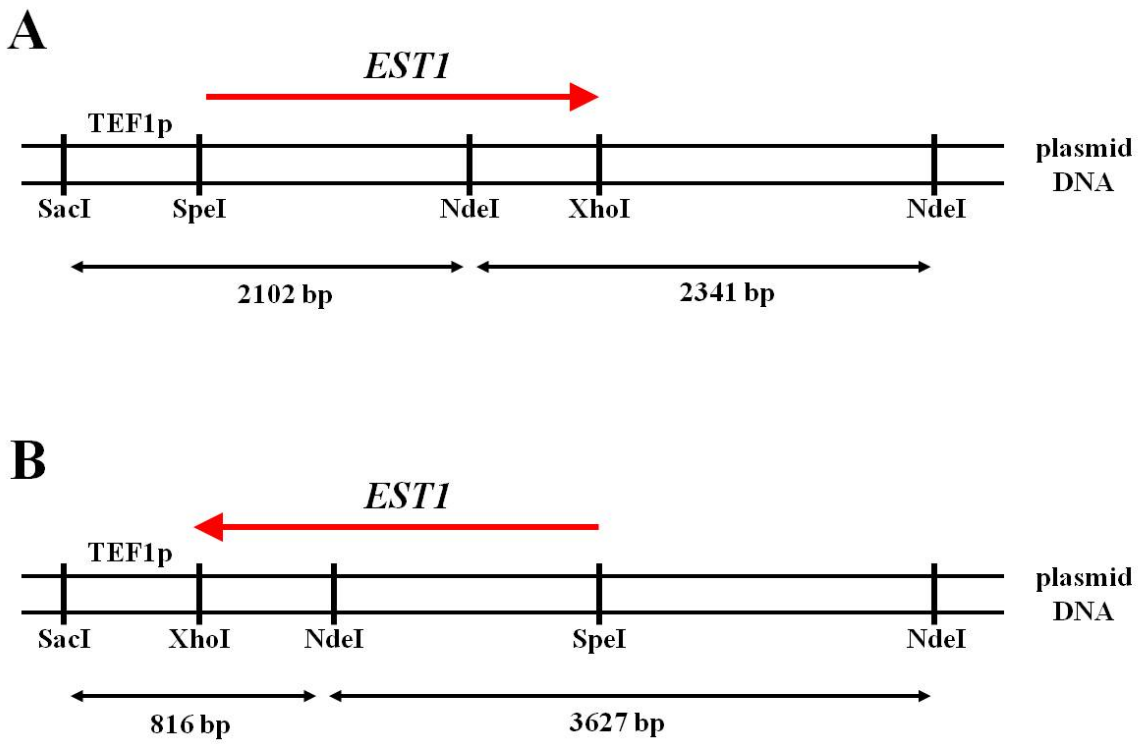

Figure 12. A. Diagram of plasmid encoding correctly oriented *EST1* gene, which produces 2102 bp and 2341 bp fragments after digestion with SacI and NdeI. B. Diagram of plasmid encoding incorrectly oriented *EST1* gene, which generates 816 bp and 3627 bp fragments after digestion with SacI and NdeI.

The SacI + NdeI digested p426TEF::EST1 isolates were subjected to gel electrophoresis on 1.2% agarose gel to confirm that the gene was inserted with correct orientation (Figure 13). The appearance of bands at ~2100 bp and ~2300 bp in lanes 2, 3, 4, and 5 indicated that the *EST1* gene was inserted into p426TEF with the correct orientation. Also, isolates 1 and 2 from the 4 preps of pLKL92Y (p426TEF::EST1) were subjected to DNA sequencing with primers T7, Tef1a1, Est1-583, and Est1-1181.

Analysis of the DNA sequences from the start codon (ATG) to the stop codon (TGA) revealed 100% identity (2100 bp) of both cloned *EST1* genes compared to the wildtype *EST1* gene listed in the Saccharomyces Genome Database (www. yeastgenome.org).

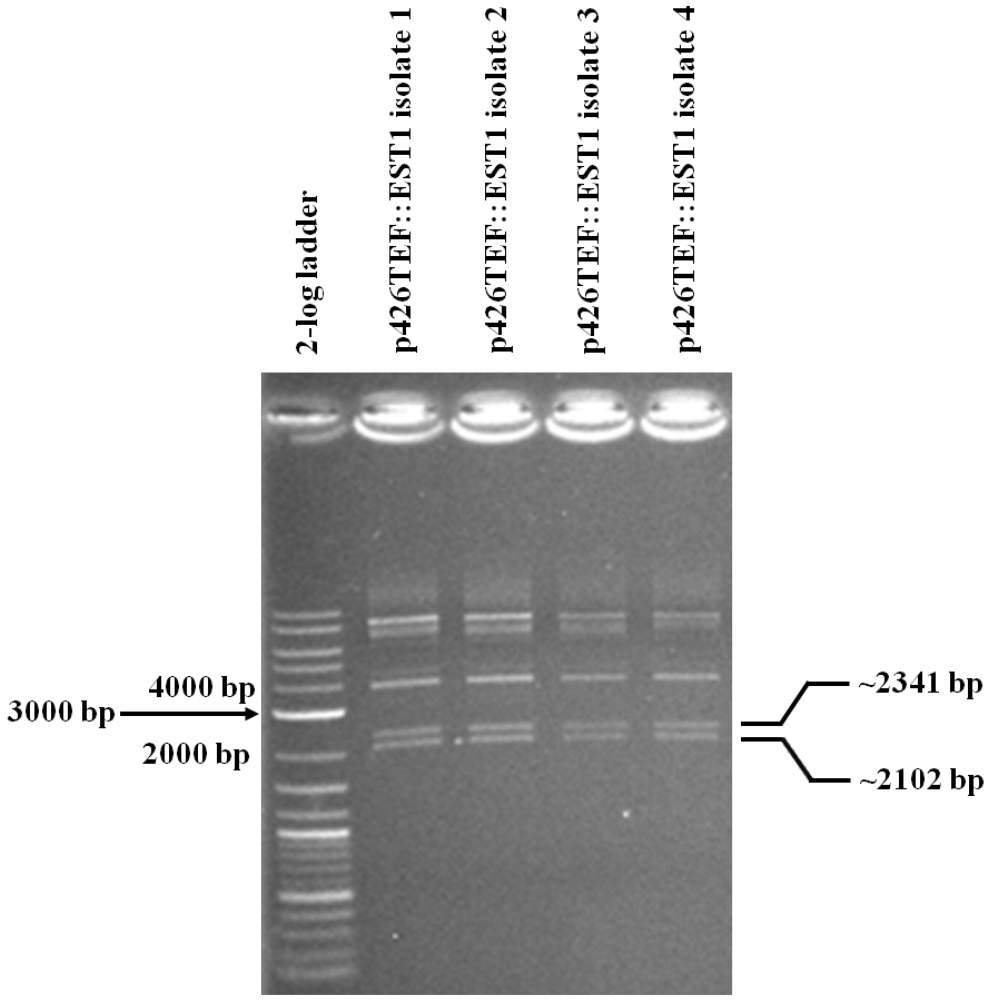

Figure 13. A 0.9% agarose gel of NdeI and SacI restriction digestions demonstrating the orientation of the gene *EST1* inserted into p426TEF.

The *EST3* gene (547 bp) was PCR amplified with primers Est3A and Est3B, followed by confirmation of the PCR product (607 bp with flanks) on an agarose gel. The *EST3* gene fragment and p426TEF vector were digested with SpeI. Prior to ligation, the

SpeI-digested p426TEF vector was subjected to a reaction with Antarctic phosphatase to prevent religation of the vector. After ligation of SpeI-digested *EST3* and p426TEF, the DNAs were transformed into 5-alpha competent *E. coli* cells and plasmid minipreps were prepared from 18 colonies. These DNAs were run on a gel and 10 of 18 migrated higher than a vector control, indicating that they had acquired an insert.

Determination of whether the gene was inserted into the p426TEF vector was performed by digesting again with SpeI, followed by gel electrophoresis on a 1.2% agarose gel (Figure 14). Lanes 2, 3, 4, 5, and 6 represent separate preps of SpeI-digested p426TEF::EST3 plasmids. Lane 3 displayed no lower band, therefore, one or more of the SpeI restriction sites were mutated. Lanes 3, 4, and 5 display bands of the expected size, but lane 2 displayed a band at ~750 bp and it was discarded.

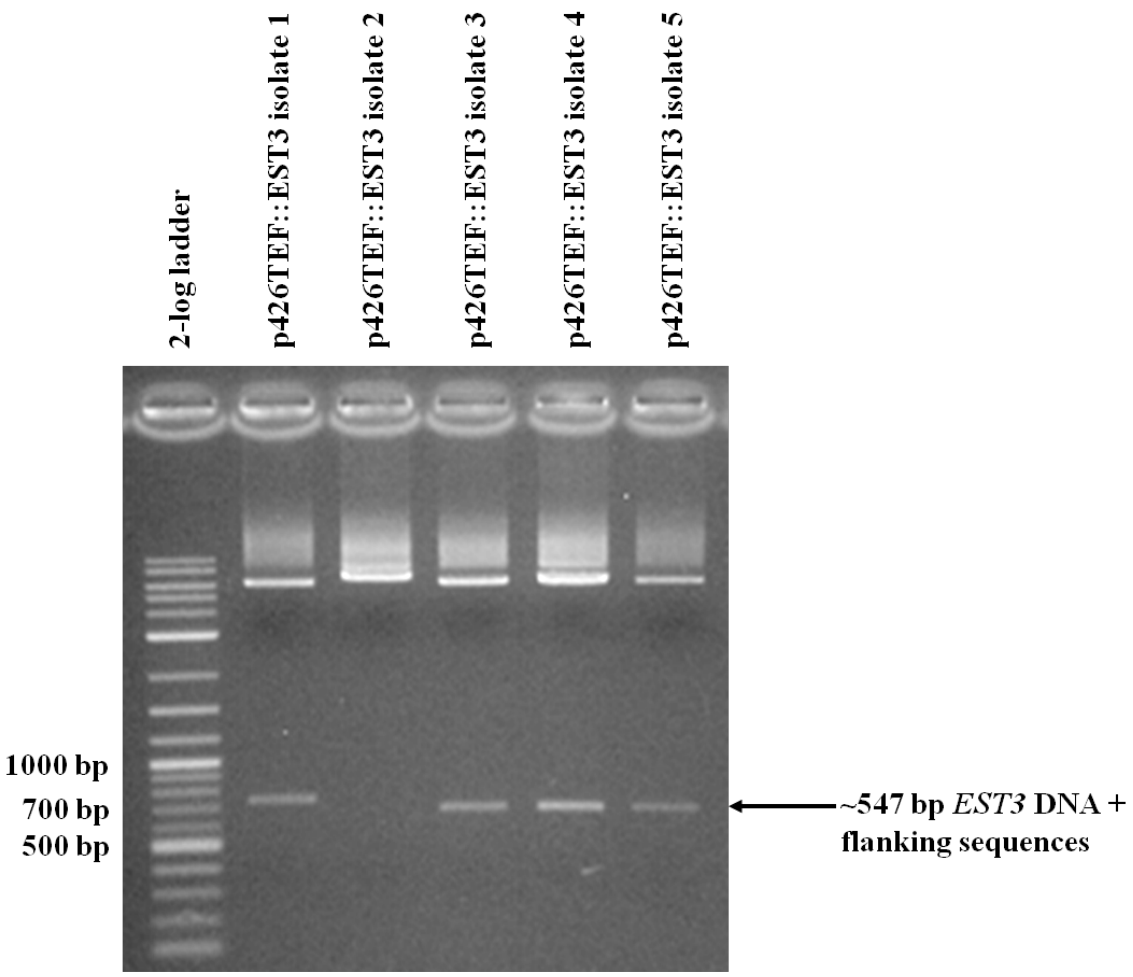

Figure 14. A 1.2% agarose gel of SpeI restriction digestions demonstrating the orientation of the gene *EST3* inserted into p426TEF.

In order to determine the orientation of the *EST3* gene inserted into the p426TEF cloning vector, a restriction digest with SacI and MscI was performed. Figure 15A illustrates an example of *EST3* digestion with correct orientation resulting in a 555 bp DNA fragment. Figure 15B illustrates an example of *EST3* digestion with incorrect orientation resulting in a 870 bp DNA fragment.

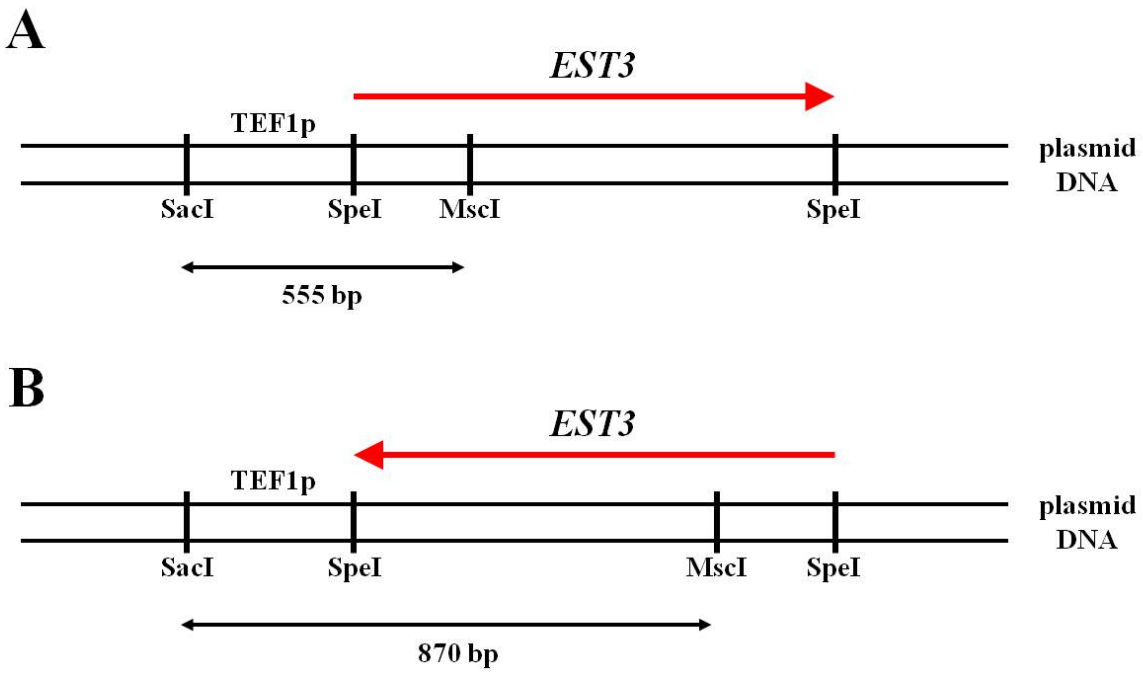

Figure 15. A. Diagram of plasmid encoding correctly oriented *EST3,* which generates a 555 bp fragment after digestion with SacI and MscI. B. Diagram of plasmid encoding incorrectly oriented *EST1*, which forms a 870 bp fragment after digestion with SacI and MscI.

The SacI and MscI digested p426TEF::EST3 isolates were subjected to gel electrophoresis on 1.2% agarose gel to confirm that the gene was inserted with correct orientation (Figure 16). The appearance of bands at ~900 bp in lanes 2 and 3 indicated that isolates 1 and 3 had the incorrect orientation. The appearance of bands at ~550 bp in lanes 4 and 5 indicated that the *EST3* gene was inserted into p426TEF with the correct orientation in isolates 4 and 5.

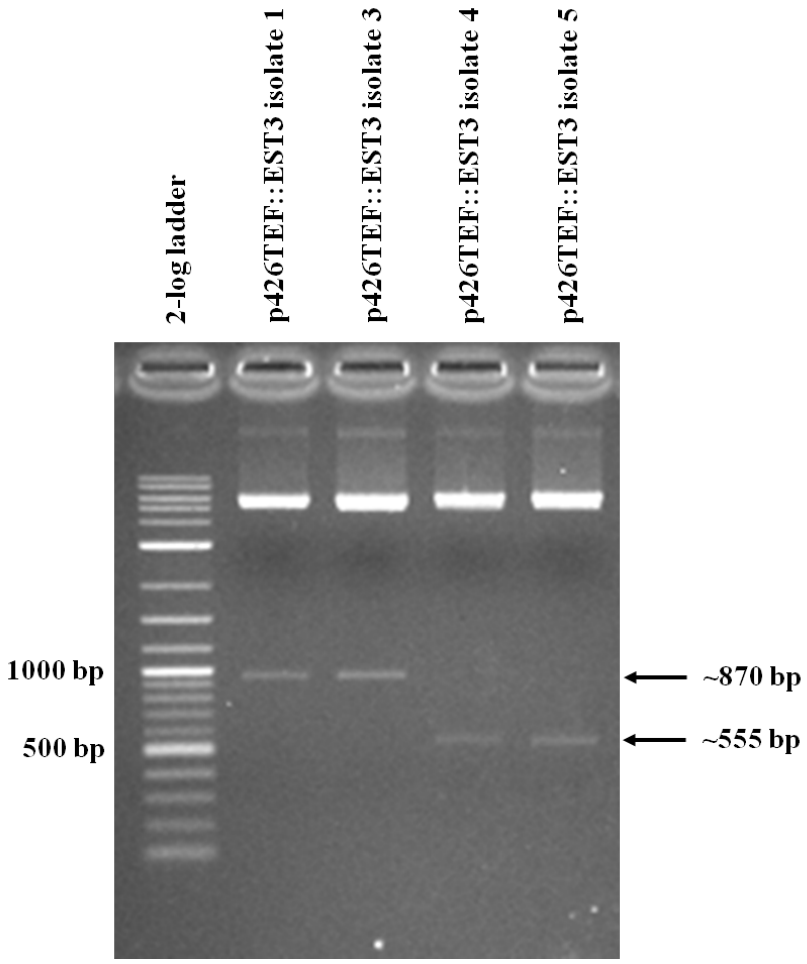

Figure 16. A 1.2% agarose gel of restriction digestions of p426TEF::EST3 plasmids demonstrating the orientation of *EST3* among different preps.

Two preps of pLKL93Y (p426TEF::EST3) were subjected to DNA sequencing with primers T7 and Tef1a1. DNA sequence analysis from the start codon (ATG) to the stop codon (TGA) revealed 100% identity compared to the wildtype *EST3* gene.

As shown earlier, overexpression of the Est2 and *TLC1* RNA subunits of telomerase can rescue the temperature-sensitivity of *yku70* mutants. pLKL83Y, pLKL92Y, and pLKL93Y (plasmids that overexpress *TLC1*, *EST1*, and *EST3*, respectively) were independently transformed into YLKL857 (*yku70Δ::G418*^{r}) cells in

order to determine whether overexpression of Est1 or another telomerase subunit, Est3, can rescue the temperature-sensitivity of *yku70* mutants. During this experiment, cells were cultured at 30 °C, harvested into water after a 2 hour incubation in YPDA broth, counted, followed by 5-fold serial dilutions into a microtiter dish. Resulting cell suspensions were then pronged onto glucose minus uracil plates and incubated at either 30 °C or 37 °C. Cells containing positive and negative control plasmids (*TLC1* gene and no *TLC1* gene, respectively) were compared to 3 independent isolates of both cells containing either *EST1* overexpression plasmid pLKL92Y (*TEF1p::EST1*) or *EST3* overexpression plasmid pLKL93Y (*TEF1p::EST3*). *TLC1* rescued the mutants, but overexpression of telomerase subunits Est1 or Est3 did not rescue the cells (Figure 17). These results do not coincide with results previously reported that *EST1* overexpression rescues *yku70* mutants (73), but do agree with previous results in the Lewis lab using a different plasmid (89). DNA sequence analysis confirmed that both *EST1* and *EST3* genes cloned into p426TEF were accurate. Thus, this experiment concluded that the telomerase subunits, Est1 and Est3, are incapable of rescuing *yku70* mutants at elevated growth temperatures.

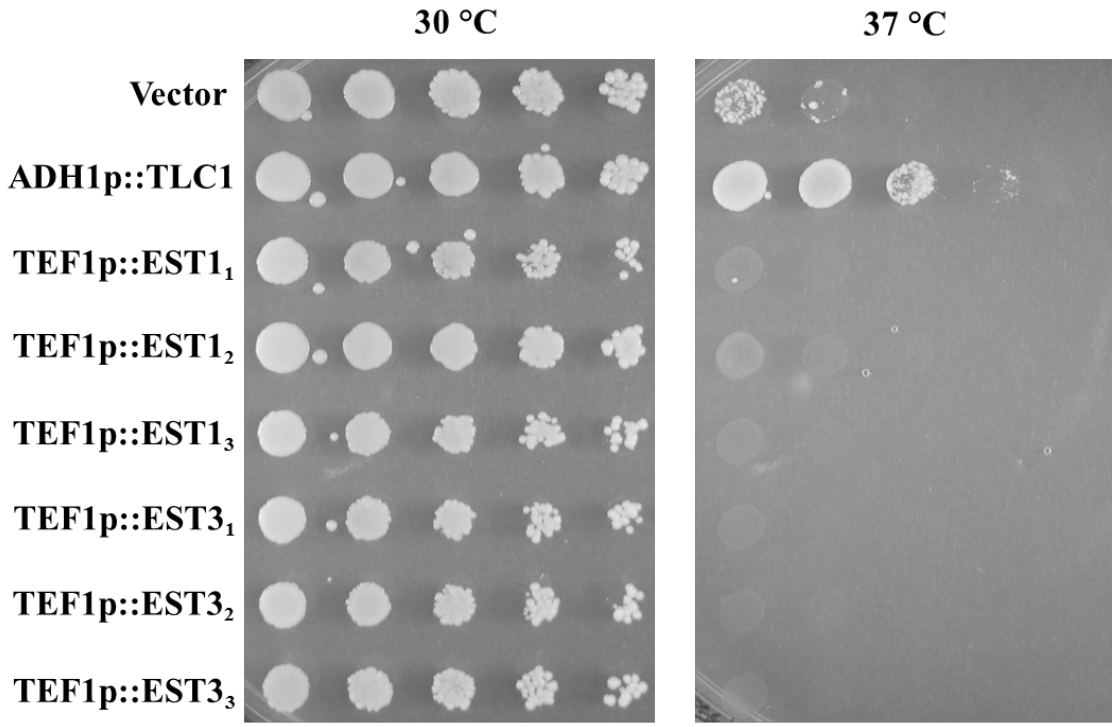

Figure 17. Rescue of *yku70* mutants at 37 °C by overexpression of *EST1* and *EST3*. Vector, pRS316 control plasmid; *TLC1*, pLKL83Y control plasmid (*ADH1p::TLC1*); *EST1*, three isolates of pLKL92Y (p426TEF + *EST1*); *EST3*, three isolates of pLKL93Y (p426TEF + *EST3*).

Est2 and *TLC1* RNA overexpression has been shown to alleviate chromosome instability of *yku70* mutants at 37 °C, thus, increasing cell viability. Other proteins involved in the repair and processing of broken chromosomes include Pif1, Srs2, and Sae2. The helicase Pif1 is known to function at the telomeres and acts as a negative regulator of telomerase (65-67). In *pif1 yku70* mutants, temperature sensitivity was suppressed compared to *yku70* mutants, which could be caused by an increase in Est2 polymerase activity (68). Sae2 is an endonuclease that works with the Mrx complex during DNA repair (62-64). Srs2 has helicase activity with 3'-5' polarity (59, 60) and research has shown that yeast cells lacking Srs2 display a shortened life span (59). The

next set of experiments was performed to further investigate the roles of Pif1, Sae2, and Srs2 in maintaining chromosome stability in *yku70* mutants. First, *yku70 pif1*, *yku70 sae2*, and *yku70 srs2* double mutants were created by disrupting the *YKU70* gene with the *YKU70* deletion plasmid pGEM4Z S-H/URA (Figure 18) in *pif1*, *sae2*, or *srs2* mutants. This plasmid was digested with HindIII and EcoRI, which released a *URA3* gene fragment with flanking sequences homologous to the termini of *YKU70*. After, transforming this digested gene fragment into cells, the regions homologous to *YKU70* associated with the chromosomal *YKU70* gene. This resulted in a recombination event, thus, disrupting the *YKU70* gene with *URA3*. Following transformation, cells were grown on glucose minus uracil plates and only cells that had undergone an integration event formed colonies.

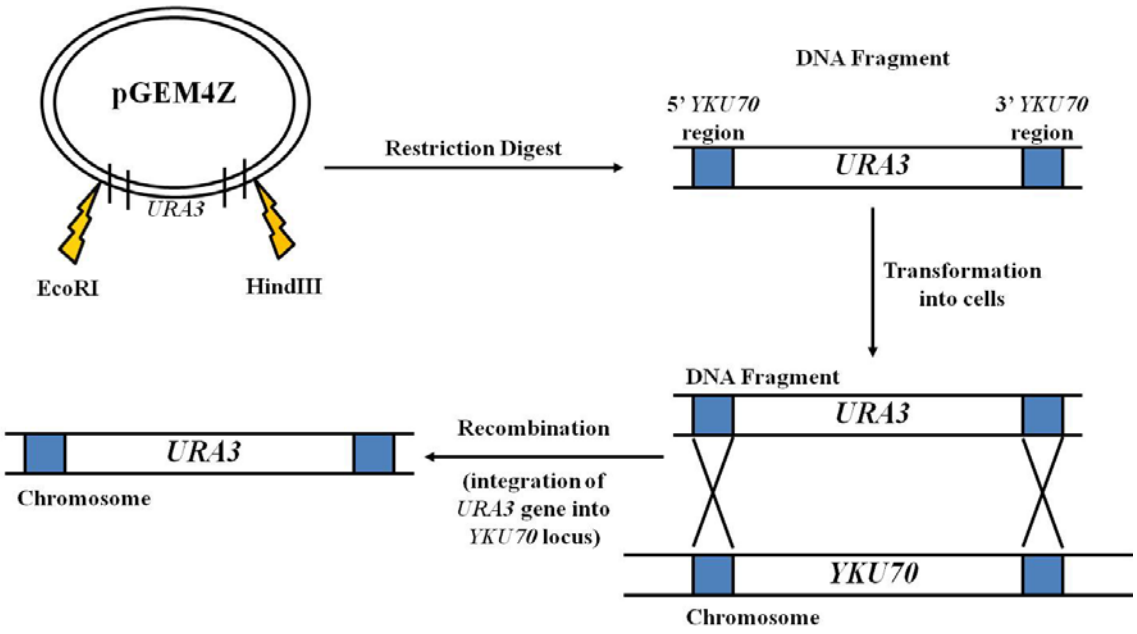

Figure 18. Illustration of the *yku70Δ*::*URA3* gene disruption method.

In order to determine whether the *URA3* gene effectively disrupted the *YKU70* gene, colonies resulting from the previous transformation were patched to fresh plates lacking uracil and grown at the normal growth temperature of 30 °C. The resulting patches were replica-plated and double-imprinted to new glucose minus uracil plates, followed by incubation at either 30 °C, 37 °C, or 39 °C for three days. Cells that could not grow at high temperatures were presumed to be *yku70*Δ*::URA3* disrupted. Confirmation of the disruption was obtained by isolating chromosomal DNA from these transformants and performing PCR using the primers Ku70a and Ku70b, followed by gel electrophoresis. An example of this analysis applied to *yku70 pif1* isolates is shown in Figure 19. The PCR product of the *URA3* gene plus the 5' and 3' flanking region is 2758 bp, whereas the wildtype *YKU70* gene with the 5' and 3' flanking region is 2223 bp. Lane 2 was the parent strain BY4742 and the appearance of a band at ~2200 bp resulted from the PCR amplification of the wildtype *YKU70* gene. Lanes 3, 4, 5, and 6 were four separate isolates of the *yku70 pif1* double mutants and the appearance of the band at ~3000 bp and disappearance of the band at ~2200 bp (*YKU70*) confirmed that *URA3* was effectively inserted into *YKU70*. The bands seen at ~1500 bp and below resulted from non-specific interactions of the primers during the PCR reactions.

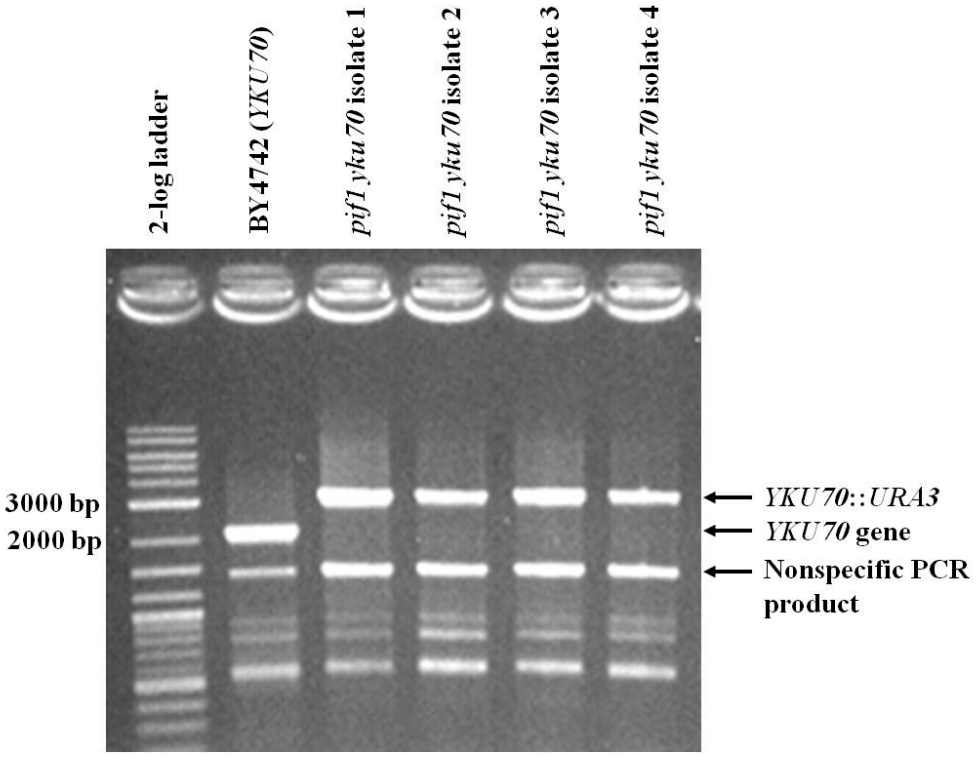

Figure 19. A 0.9% agarose gel assessing *yku70Δ*::*URA3* disruption in a *pif1Δ::G418*^{r} mutant strain.

Also analyzed were *yku70* disruptions resulting in *yku70 srs2 and yku70 sae2* double mutants, which were confirmed by PCR using the primers Ku70a and Ku70b as described above (Figure 20). Lane 2 was the parent strain BY4742. Lanes 3, 4, and 5 were three separate isolates of the *yku70 srs2* double mutants and the appearance of the band at ~3000 bp and disappearance of the band at ~2200 bp (*YKU70*) in lane 3 and 4 confirmed that *URA3* effectively replaced *YKU70*. However, isolate 3 in lane 5 did not show either band, so the isolate was discarded. The band at ~3000 bp and absence of the band at ~2200 in lanes 6 and 7 confirmed the *yku70*Δ*::URA3* disruption in two isolates of

the *sae2*Δ*::G418*^{r} strain. Again, the bands seen at ~1500 bp and below resulted from non-specific interactions of the primers during the PCR reactions.

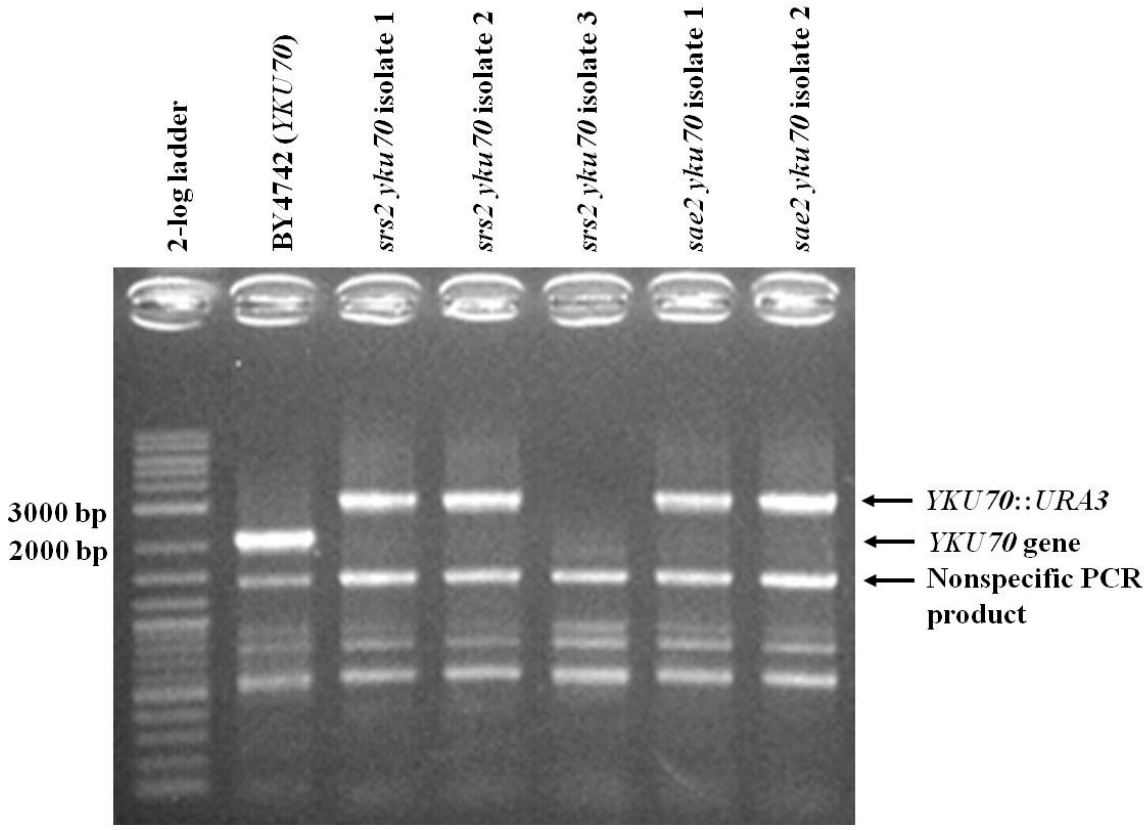

Figure 20. A 0.9% agarose gel assessing *yku70Δ*::*URA3* disruption in *srs2Δ::G418*^{r} and *sae2Δ::G418*^{r} mutant strains.

After the *yku70*Δ*::URA3* disruptions were confirmed via PCR in *pif1*, *sae2*, and *srs2* cells, a preliminary analysis was conducted to assess the effect of inactivation of *SRS2, SAE2,* and *PIF1* on *yku70* temperature sensitivity (Figure 21). Double mutant and *yku70* mutants were grown on glucose minus uracil, harvested, counted, and pronged to fresh glucose minus uracil plates. These plates were then incubated at either 30 °C or 37

°C for 3-4 days. Analysis of the plates revealed that *pif1 yku70* mutants partially alleviated the temperature sensitivity phenotype of *yku70* mutants. This is likely to occur due to the absence of negative regulation by Pif1 on Est2 polymerase at the telomeres. The *yku70 sae2* and *yku70 srs2* double mutants displayed a temperature sensitivity phenotype typical of *yku70* mutants.

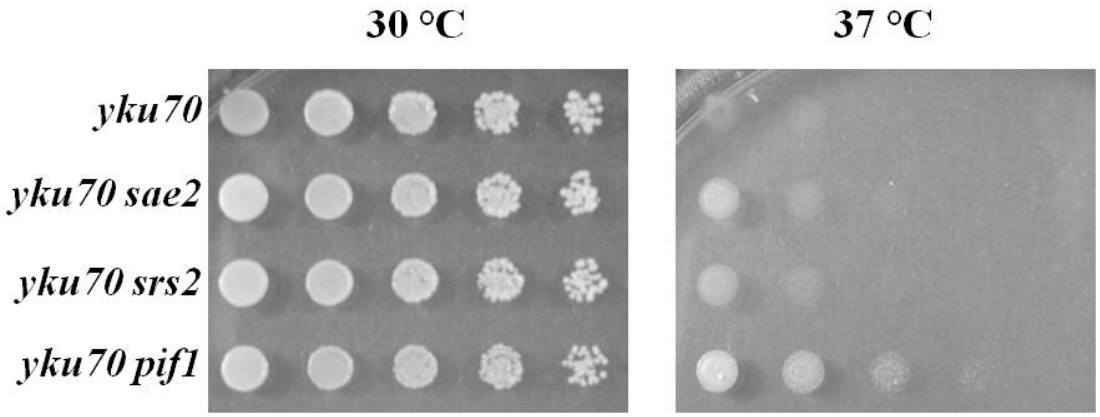

Figure 21. Determination of temperature sensitivity at 37 °C after *YKU70* deletion in *sae2, srs2,* and *pif1* mutant strains.

Supraphysiological levels of Est2 polymerase and *TLC1* RNA have already been shown to rescue *yku70* mutants, so the next experiment was to determine whether *yku70* cells lacking the proteins Sae2 or Srs2 could be rescued at elevated growth temperatures by Est2 or *TLC1* overexpression. To assess this question, *yku70*, *yku70 sae2*, *yku70 srs2*, and *yku70 pif1* were independently transformed with pRS315 (*CEN/ARS LEU2*), pVL999 (*2μ LEU2 ADH1p::EST2*), and pLKL64Y (*2μ LEU2 ADH1p::TLC1*). Transformant colonies were grown on glucose minus leucine at 30 °C, harvested, counted, and pronged

to fresh glucose minus leucine plates. These plates were then incubated at either 30 °C or 37 °C for 2-3 days. Analysis of the plates showed that Est2 overexpression in the *yku70* control strain resulted in strong rescue compared to vector and *TLC1* RNA resulted in weak rescue. Rescue by *TLC1* RNA overexpression in *yku70 sae2* and *yku70 srs2* was weaker than in *yku70* mutants, but rescue by Est2 polymerase was strong and similar to its effects in the single mutants. In *yku70 pif1* double mutants, both Est2 and *TLC1* RNA overexpression showed stronger rescue than in the single mutants (Figure 22). These results indicate that, whatever the mechanism involved in stabilization of the telomeres by Est2 and *TLC1* overexpression, it does not require the enzymatic activities of Sae2, Srs2, or Pif1.

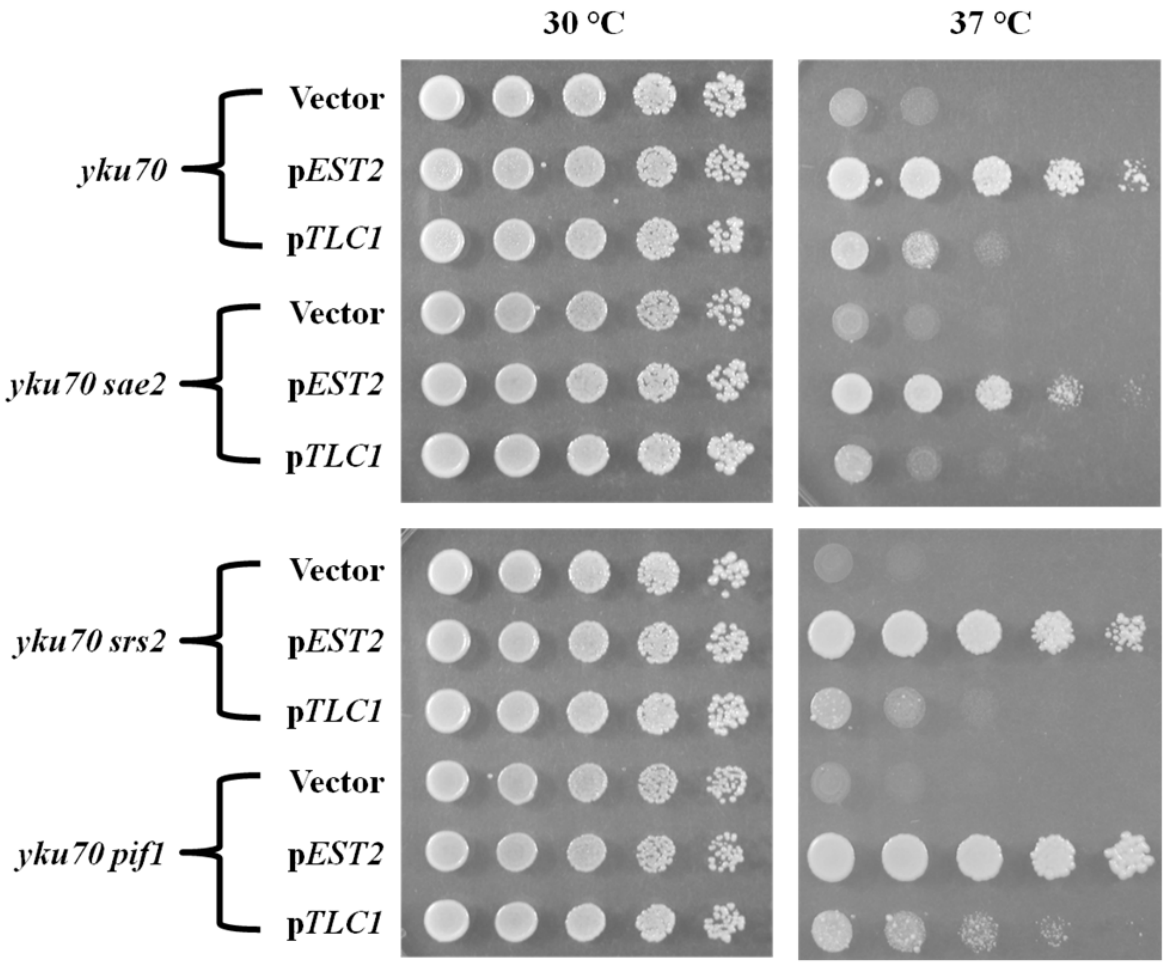

Figure 22. Rescue of *yku70*, *yku70 sae2*, *yku70 srs2*, and *yku70 pif1* mutants at 37 °C by overexpression of *EST2* polymerase and *TLC1* RNA.

The experiments described here have expanded upon previous findings of the students Brian Wasko and Cory Holland in the Lewis lab (77, 90). Several results from all three projects are combined and summarized in Tables 3, 4, and 5. Results in Table 3 show that among several genes encoding nucleases, helicases, and telomere chromatin proteins, only *exo1*, *rif1*, and *pif1* mutations alleviated the killing of *yku70* mutants at high temperatures. These results are logical because inactivation of *EXO1* removes a

nuclease that degrades telomeres and inactivation of *rif1* and *pif1* remove inhibition of telomerase, freeing it up to stabilize the telomeres.

Table 3. Survival of *yku70* double mutants at high temperatures.

Double mutant	Alleviation of the death of *yku70* cells at high temperature	Reference
yku70 rad1	No	77
yku70 rad10	No	77
yku70 mms4	No	77
yku70 mus81	No	77
yku70 rad17	No	77
yku70 rad24	No	77
yku70 rif2	No	77
yku70 sir2	No	77
yku70 sir3	No	77
yku70 sir4	No	77
yku70 mlp1	No	77
yku70 mlp2	No	77
yku70 mlp1 mlp2	No	77
yku70 exo1	**Yes**	77, 26
yku70 rif1	**Yes**	77, 26
yku70 sae2	No	This work
yku70 srs2	No	This work
yku70 pif1	Yes (Weak)	This work, 76

Table 4 summarizes the ability of *EST2* and *TLC1* overexpression to rescue *yku* single and double mutant strains. Both telomerase subunits improved survival at high temperatures, though in some cases the rescue by *TLC1* RNA was quite modest. In Table 5 all of the telomerase- and telomere-associated proteins that have been overexpressed in

these studies are listed. Among these, only *EST2* and *TLC1* RNA were successful, including two mutants of the Est2 polymerase that lack catalytic activity (Est2-D530A and Est2-D670). These results suggest that Est2 protein and *TLC1* RNA are uniquely able to stabilize the telomere cap complexes of cells lacking Ku. Furthermore, the results obtained with the inactive polymerases indicate that rescue does not involve extension of the short telomeres of *yku70* mutants by the polymerase function of telomerase. Instead, the mechanism is likely to involve a strengthening of protein:protein interactions at telomere ends, reinforcing the cap structure and protecting the DNA ends from nucleases.

Table 4. *EST2* and *TLC1* rescue of *yku70* mutants at high temperatures.

Mutant	Rescue by *EST2*	Rescue by *TLC1*
yku70	Yes	Yes
yku80	Yes	Yes
yku70 yku80	Yes	Yes
yku70 rif1	Yes (39 °C)	Yes (39 °C)
yku70 rif2	Yes	Yes
yku70 sir3	Yes	Yes
yku70 sir2	Yes	(Weak)
yku70 sir4	Yes	Yes
yku70 mlp1	Yes	Yes
yku70 mlp2	Yes	Yes
yku70 mlp1mlp2	Yes	Yes
yku70 sae2	Yes	(Weak)
yku70 srs2	Yes	(Weak)

Table 5. Rescue of *yku70* mutants at 37 °C.

Overexpressed gene	Rescue of *yku70* cells	Reference
TLC1	Yes	77
EST2	Yes	77
EST2-D530A	Yes	90
EST2-D670	Yes	90
TEN1	No	90
STN1	No	90
CDC13	No	90
RIF1	No	LKL unpublished
RIF2	No	LKL unpublished
SIR3	No	LKL unpublished
SIR4	No	LKL unpublished
EST1	No	This work
EST3	No	This work

During the course of this project it was necessary to purify DNA out of many different yeast strains, including wildtype BY4742 cells and several *yku70* mutant strains. A previously published protocol by the Lewis lab (86) was used for rapid purification of the DNA. However, this method and other previously described methods generate yeast chromosomal DNA that also has a large quantity of small contaminating RNA molecules in it. The RNAs were digested with RNase A at the end of the DNA purification procedure (a standard procedure in most DNA purification protocols), reducing most of the molecules to small oligonucleotides. During the course of RNA digestions in the current project, it was apparent that some of the RNA was resistant to standard digestion methods with RNase A and produced two higher molecular weight bands during gel electrophoresis. In the next part of this thesis project, several experiments were

performed in an effort to develop a procedure for the degradation and removal of contaminating RNA molecules.

In order to assess the RNA contamination problem, gel electrophoresis was performed initially on yeast chromosomal DNA preps that had not been treated with RNase to determine the relative quantity and molecular weight of the RNA molecules (Figure 23A). The upper bands above 10 kb in lanes 2 and 3 represent the chromosomal DNA and the bands seen from ~6000 bp and below represent the contaminating RNA. The band seen in lanes 2 and 3 at approximately 3000 bp most likely represents the 26S rRNA, which is a component of the 60S ribosomal subunit. The band at about 200 bp is most likely a combination of the 5.8S rRNA and 5S rRNA, which are also components of the 60S subunit. The band at about 1500 bp most likely represents the 18S rRNA, which is a component of the 40S ribosomal subunit (Saccharomyces Genome Database; www.yeastgenome.org).

These samples were subjected to a standard digestion by RNase A for 30 minutes at 37 °C, followed by analysis via gel electrophoresis (Figure 23B). It is important to note that this gel and all subsequent gels were run at high voltages with short run times (~15 minutes) to minimize diffusion of small RNA molecules. In lanes 2 and 3, the upper band represents the chromosomal DNA and there are two bands at the bottom of the gel image representing undigested RNA. Incubation with more RNase A enzyme or for longer times did not improve results (data not shown). RNase A is a pancreatic endoribonuclease that specifically cuts single-stranded RNA at the 3’ end of pyrimidines, producing small oligomers and single nucleotides. The two RNA bands remaining in

Figure 23B are likely to be resistant to RNase A because they have retained secondary and tertiary structure and represent mostly double-stranded RNA.

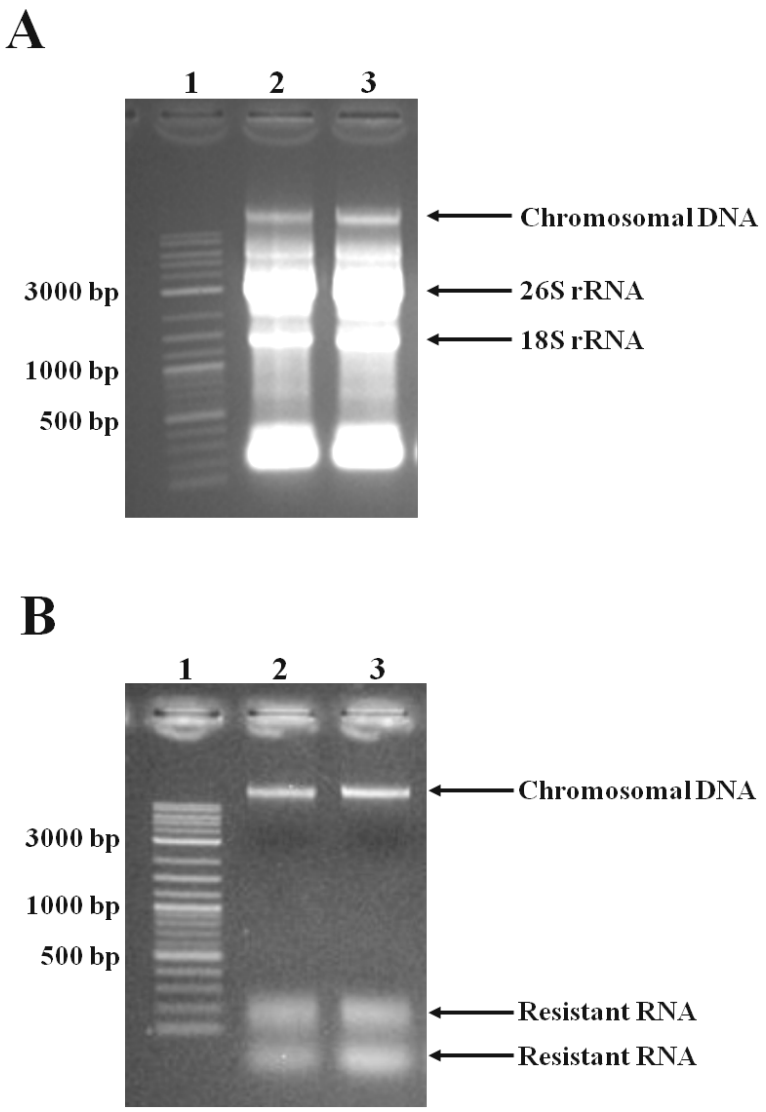

Figure 23. A. Gel electrophoresis of yeast chromosomal DNA preps prior to RNase A digestion on a 1.0% agarose gel. *Lane 1*, 2-log ladder; *lane 2*, yeast chromosomal DNA purification (sample 1); *lane 3*, yeast chromosomal DNA purification (sample 2). **B. Gel electrophoresis of yeast chromosomal DNA purifications after RNase A digestion for 30 minutes at 37 °C on a 1.0% agarose gel.** *Lane 1*, 2-log ladder; *lane 2*, yeast chromosomal DNA purification (sample 1); *lane 3*, yeast chromosomal DNA purification (sample 2).

These experiments determined that contaminating RNAs of about 50-200 bp in size (relative to the dsDNA standards) were resistant to digestion by RNase A. The next set of experiments was performed in an attempt to separate the RNA molecules from the yeast chromosomal DNA using either the QIAprep Spin Miniprep or CHROMA SPIN-1000 columns. Qiagen spin columns are anion-exchange columns that bind plasmid DNA efficiently, but not small nucleic acids like PCR primers. CHROMA SPIN-1000 columns contain a proprietary gel chromatography resin that fractionates molecules based on size. Yeast chromosomal DNA was purified from wildtype BY4742 cells using the protocol described before (86), followed by RNase A digestion for 30 minutes at 37 °C and gel electrophoresis analysis. Figure 24A shows the yeast chromosomal DNA preps following application to Qiagen miniprep columns. Lane 2 is the control and shows the DNA prep with the contaminating RNA before being applied to the column. Lanes 2 and 3 show independent DNA preps after being applied to the columns and eluted with ddH_2O. In both samples, there was a substantial loss in chromosomal DNA, which most likely results from these columns being designed for plasmid DNA, not chromosomal DNA. Also, the higher molecular weight RNA was not removed whereas the lower molecular weight RNA was successfully removed.

Figure 24B shows the yeast chromosomal DNA preps following application to CHROMA SPIN-1000 columns. These columns were specifically designed for plasmid or chromosomal DNA (> 1350 bp) purification, and according to the manufacturer, greater than 99% of DNA or RNA molecules less than 300 bp should be removed. Lane 2 is the control and shows the DNA prep with the contaminating RNA before being applied to the column. Lanes 2 and 3 shows the DNA preps after being applied to the

columns. Again, both samples resulted in loss of DNA, though the amount of loss was not as severe as with the Qiagen columns. The CHROMA SPIN columns were able to remove more RNA compared to the Qiagen spin columns, but a small fraction of the higher molecular weight RNA was still present.

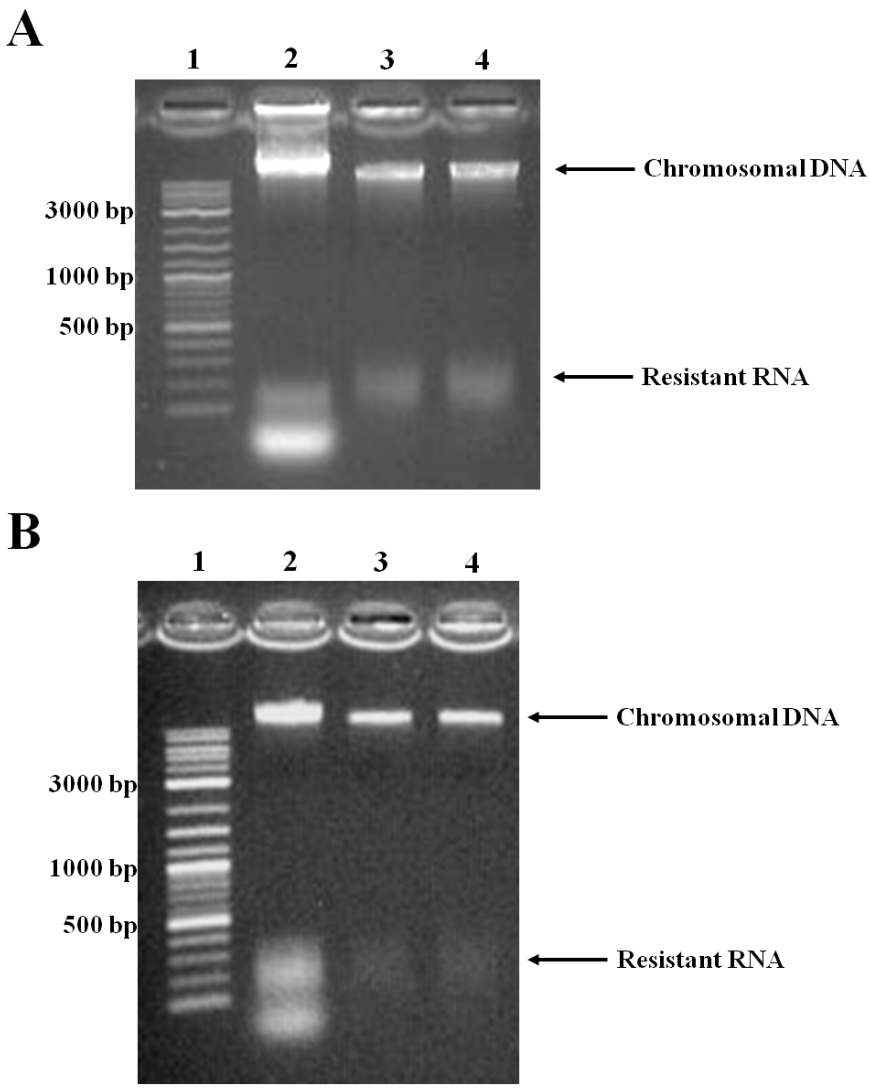

Figure 24. A. Gel electrophoresis of yeast chromosomal DNA preps using the QIAprep Spin Miniprep column on a 1.4% agarose gel. *Lane 1*, 2-log ladder; *lane 2*, yeast chromosomal purification (sample 1); *lane 3*, yeast chromosomal purification (sample 2**). B. Gel electrophoresis of yeast chromosomal DNA purifications using the CHROMA SPIN-1000 columns on a 1.0% agarose gel.** *Lane 1*, 2-log ladder; *lane 2*, yeast chromosomal purification (sample 1); *lane 3*, yeast chromosomal purification (sample 2).

Both types of columns were unsuccessful at removing higher molecular weight RNA molecules. Thus, the next set of experiments aimed to further digest the resistant RNA molecules into smaller fragments. These RNA molecules are resistant to digestion by RNase A, so another enzyme, RNase $I_{f,}$ was employed. RNase I_f is an *E. coli* endoribonuclease that cleaves ssRNA, but unlike RNase A, can cleave after all four nucleotides. Yeast chromosomal DNA was purified from wildtype BY4742 cells in the same manner as previously stated, followed by RNase I_f digestion for 30 minutes at 37 °C and gel electrophoresis analysis (Figure 25A). Lanes 2 and 3 show that the RNA was degraded. However, there appeared to be non-specific nuclease degradation of the DNA based on the smearing down the lanes and strong fluorescent band at the bottom of the gel. In some experiments, RNase I_f was able to degrade the RNA somewhat further (images not shown), but this result was not consistent and Figure 25A was the typical outcome generated from this enzyme. It is not clear why some chromosomal DNA degradation was frequently observed with the enzyme.

In an effort to reduce the resistance of the small RNAs, heat was applied to the samples prior to digestion with RNase A to destabilize the RNA structure and create more ssRNA. After the purification of chromosomal DNA, the samples were incubated at 65 °C for 5 minutes, followed immediately by digestion with RNase A for 30 minutes at 37 °C and gel electrophoresis analysis (Figure 25B). Lane 2 is the control in which the chromosomal DNA prep was subjected to the standard RNase A procedure. Lane 3 shows the effect of heating the samples prior to digestion. The levels of each of the RNAs were reduced. This result demonstrated that heating the RNAs reduced their resistance to digestion, which could be caused by making the RNAs more single-stranded

and therefore a more efficient substrate for the enzyme. Note that several heating temperatures were tested, including 65, 70, 75, and 80 °C, but best results were observed at ~65-70 °C.

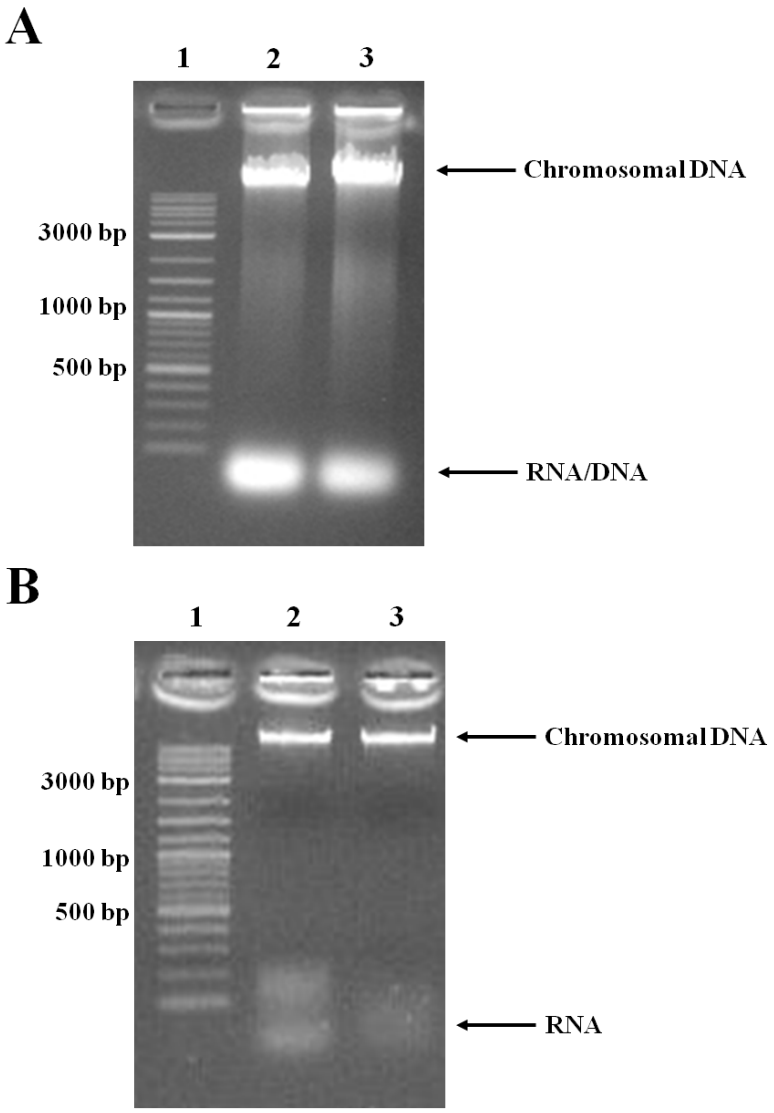

Figure 25. A. Gel electrophoresis of yeast chromosomal DNA preps after RNase I_f digestion for 30 minutes at 37 °C on a 1.4% agarose gel. *Lane 1*, 2-log ladder; *lane 2*, yeast chromosomal purification (sample 1); *lane 3*, yeast chromosomal purification (sample 2). **B. Yeast chromosomal DNA preps after heating the samples for 5 minutes at 65 °C, followed by RNase A digestion for 30 minutes at 37 °C and gel electrophoresis on a 1.4% agarose gel.** *Lane 1*, 2-log ladder; *lane 2*, yeast chromosomal purification (no heat); *lane 3*, yeast chromosomal purification (with heat).

To further investigate the degradation of contaminating RNA molecules, new RNase enzymes were tested. The previous enzymes, RNase A and RNase I_f are both specific for single-stranded RNA. Thus, it was considered that the resistance of RNA molecules could be caused by the fact that they exist in a double-stranded state, which could explain why the addition of heat prior to RNase A digestion reduced this resistance. The next experiment utilized the double-stranded specific enzyme RNase V1, in addition to an RNase Cocktail (from Invitrogen), which is a mixture of RNase A and RNase T1 (single-stranded specific). After the purification of chromosomal DNA as before, different samples were subjected to various conditions of RNase digestion for 30 minutes at 37 °C, followed by analysis via gel electrophoresis (Figure 26). Lane 2 was the control and showed the standard digestion by RNase A, which resulted in two bands at the bottom of the gel representing the RNA. Digestion with RNase Cocktail is shown in lanes 3 and 4, which resulted in increased digestion of the higher molecular weight RNA band. Lane 5 shows digestion with RNase V1 in the presence of the "RNA structure buffer" provided by the manufacturer; however, this resulted in degradation of chromosomal DNA. Lane 6 shows digestion with dsRNA-specific RNase V1 alone without the RNA structure buffer, which resulted in degradation of the RNA to a maximum of ~200 bp, similar to that of RNase Cocktail. Lane 7 shows digestion with both RNase V1 and RNase Cocktail together. This resulted in degradation of the RNA even further, but a large fraction of the RNA remained. Lane 8 shows digestion with RNase V1 and RNase Cocktail together in RNA structure buffer. This combination resulted in almost complete digestion of the chromosomal DNA and the RNA. Based on these results, the combination of both RNase V1 and RNase Cocktail were able to digest

the higher molecular weight RNA molecules to the smallest fragments, but the reaction had to be done without adding the manufacturer's buffer to prevent DNA degradation.

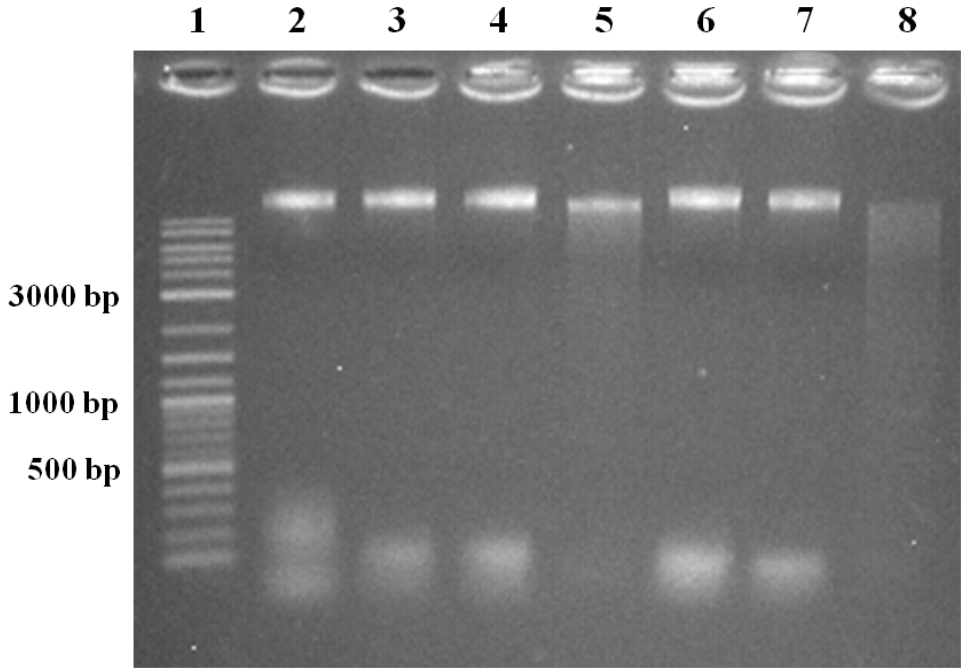

Figure 26. A 1.0% agarose gel electrophoresis analysis of yeast chromosomal DNA purifications digested with RNase Cocktail, RNase V1, or both. *Lane 1,* 2-log ladder; *lane 2,* digestion with RNase A; *lane 3,* digestion with RNase Cocktail; *lane 4,* digestion with RNase Cocktail; *lane 5,* digestion with RNase V1 in the presence of RNA structure buffer; *lane 6,* digestion with RNase V1; *lane 7,* digestion with RNase Cocktail and RNase V1; *lane 8,* digestion with RNase Cocktail, RNase V1, and RNA structure buffer.

Previously it was shown that heating the chromosomal DNA preps prior to digestion with RNase A can reduce the resistance of the RNAs. Also, using a mixture of RNase Cocktail and RNase V1 appeared to be more efficient in the digestion of RNA molecules compared to RNase A alone. Based on the previous analyses, the next set of

experiments aimed to combine the variable of heat with the combination of RNase Cocktail and RNase V1 in an attempt to further degrade the contaminating RNA molecules into smaller oligomers or monomers. To test this, samples were subjected to digestion with RNase Cocktail and RNase V1 for 30 minutes at 37 °C with or without a pre-incubation period for 5 minutes at 65 °C, followed be gel electrophoresis analysis (Figure 27). Lane 2 was the control and showed the standard digestion by RNase A. Lanes 3 and 4 show RNA digestions without pre-heating of the chromosomal DNA preps and the gel image indicated that the RNA was degraded to a smaller size. Lanes 5 and 6 show digestions with pre-heating of the chromosomal DNA preps and the gel image indicated that the RNA was degraded even further. Thus, heating the samples prior to the RNase reaction was able to increase the efficiency of the enzymes, which is possibly due to denaturation of the RNA molecules' secondary structure, but it was still not possible to degrade all of the RNA.

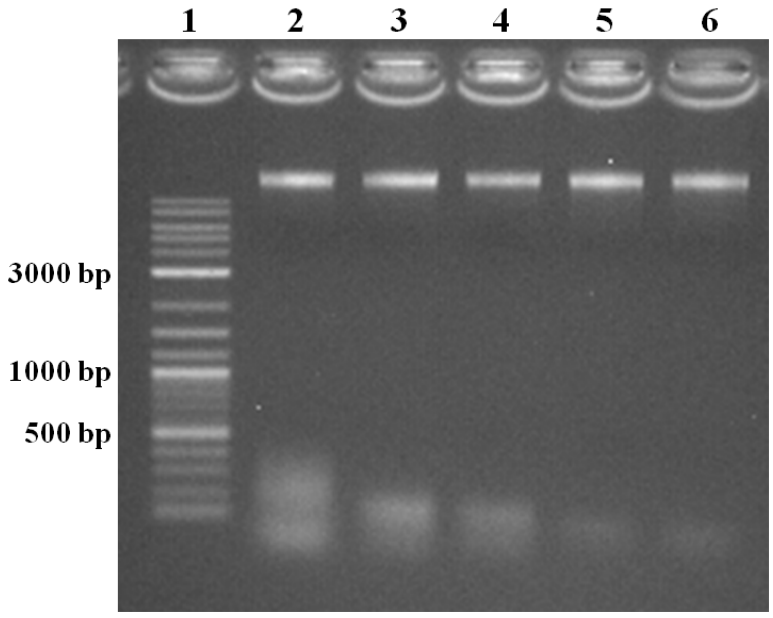

Figure 27. A 1.0% agarose gel electrophoresis analysis of yeast chromosomal DNA preps digested with RNase Cocktail and RNase V1 for 30 minutes at 37 °C with and without a pre-heating period for 5 minutes at 65 °C. *Lane 1*, 2-log ladder; *lane 2*, digestion with RNase A; *lane 3*, digestion with RNase Cocktail and RNase V1 (no heat); *lane 4*, digestion with RNase Cocktail and RNase V1 (no heat); *lane 5*, digestion with RNase Cocktail and RNase V1 (with heat); *lane 6*, digestion with RNase Cocktail and RNase V1 (with heat).

The previous RNase experiments enabled the development of a method to degrade the resistant RNA molecules to smaller sizes. However, a small amount of contaminating RNA was still present in the yeast chromosomal DNA preps. The next series of experiments addressed separation of the chromosomal DNA from the RNA by alcohol precipitation. The RNA/protein removal method described by Feliciello and Chinali was employed to purify BY4742 chromosomal DNA from RNA (87). The published protocol involved the addition of 1.2 volumes of an 88% isopropanol/0.2 M KOAc mixture to a DNA solution, followed by incubation for 10 minutes at room temperature and centrifugation for 5 minutes at 16,000 x g. The resulting DNA pellet

was then resuspended in ddH_2O with the original volume of the starting DNA solution. Initial efforts to use this approach to remove the resistant RNAs revealed that this method was not efficient at removing all of the RNA (data not shown). Thus, modifications were tested to improve the capabilities of the method for removing RNA from chromosomal DNA.

In an attempt to improve this method, the first set of parameters tested was the length of centrifugation times after the addition of the 88% isopropanol/0.2 M potassium acetate solution. It was thought that by decreasing the time of centrifugation, there would be less RNA precipitation due to its smaller fragment size compared to chromosomal DNA. Following yeast chromosomal DNA preparation as before, the samples were subjected to a standard RNase A digestion without a pre-heating period. The samples were then subjected to the Feliciello and Chinali protocol with centrifugation for either 1, 2, or 5 minutes, followed by gel electrophoresis analysis (Figure 28A). Lane 2 was the control and showed the standard digestion by RNase A, which resulted in large quantities of resistant RNA molecules at the bottom of the gel. Lane 3 represents the standard RNA/protein removal method described by Feliciello and Chinali with a centrifugation time of 5 minutes. Lanes 4 and 5 represent the same RNA removal method with the time of centrifugation decreased to 2 and 1 minutes, respectively. The gel image showed little variability in the results between the different times of centrifugation, following the RNA removal method. In each case, smaller RNA fragments were removed, but the higher molecular weight RNA remained in the solution. Some of the RNA appeared to migrate at a higher molecular weight compared to the control, which most likely resulted from the transition of RNA from a more compact structure to a more relaxed state, which may

have a larger average size. Centrifugation for 2 minutes was used in subsequent experiments based on the ability to save time during chromosomal DNA purifications.

The next set of parameters tested was the concentration of potassium acetate (KOAc) in the isopropanol solution used for precipitation. The purpose of salt in alcohol precipitation of nucleic acids is to neutralize the negative charges on the sugar phosphate backbone, thus, making the molecules more capable of aggregating closely to precipitate out of solution. Based on this principle, a lower concentration of KOAc could selectively remove only larger molecules of nucleic acids and leave smaller fragments in solution. Samples were subjected to RNA removal with 88% isopropanol solutions containing KOAc concentrations of 0.05, 0.1, 0.15, and 0.2 M, followed by gel electrophoresis analysis (Figure 28B). Lane 1 was the control and represents the standard digestion by RNase A. The following lanes used this DNA solution digested with RNase A for experimentation. Lanes 2, 3, 4, and 5 show precipitations with 0.2, 0.15, 0.1, and 0.05 M KOAc. Yields of the upper DNA band remained high, but the RNA bands decreased with the lowering of KOAC concentrations. Lane 5 represents the RNA removal method with 0.05 M KOAc and the gel image indicated the least amount of RNA precipitation; however, there appeared to be some loss of chromosomal DNA. Thus, it was concluded that 0.1 M KOAc was optimal for removing the most RNA molecules with the minimal depletion of the chromosomal DNA. However, this approach did not succeed in removing all of the contaminating RNA.

The final set of parameters examined was the addition of different volumes of the isopropanol/KOAc solution. By lowering the amount of alcohol, the solubility of small molecules will be increased and they will not precipitate out. Larger chromosome-sized

molecules will still aggregate into insoluble complexes. Following yeast chromosomal DNA purification, samples were subjected to a standard RNase A digestion without a pre-heating period. The samples were then subjected to the RNA removal method with addition of 0.4, 0.5, 0.6, and 1.2 volumes of the 88% isopropanol/0.1 M KOAc solution, followed by gel electrophoresis analysis (Figure 28C). Lane 2 was the control and represents the standard digestion by RNase A. Lane 3 represents the addition of 1.2 volumes of the isopropanol/KOAc solution, followed by centrifugation and the gel image indicated that a small quantity of RNA molecules was removed. Lanes 4, 5, and 6 represent the addition of 0.6, 0.5, and 0.4 volumes of the isopropanol solution, respectively. Results shown in lane 4 indicated that 0.6 volumes was able to remove all of the detectable RNA with no apparent loss of chromosomal DNA.

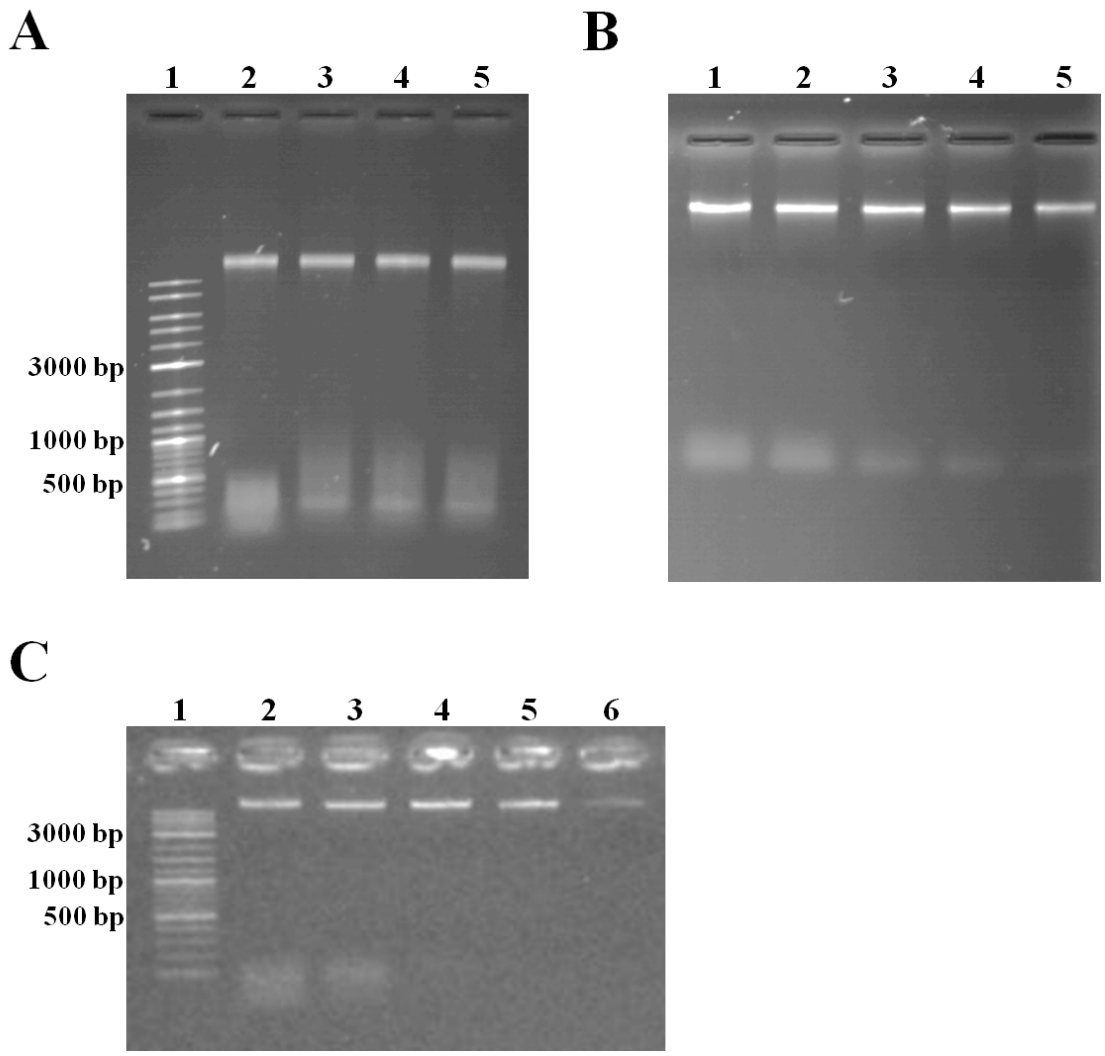

Figure 28. A. Gel electrophoresis of the RNA removal method with different centrifugation times. *Lane 1*, 2-log ladder; *lane 2*, control DNA subjected to RNase A only; *lane 3*, 5 minute centrifugation; *lane 4*, 2 minute centrifugation; *lane 5*, 1 minute centrifugation**. B. Gel electrophoresis of the RNA removal method with different concentrations of KOAc on a 1.4% agarose gel.** *Lane 1*, control DNA subjected to RNase A only; *lane 2*, 0.2 M KOAc; *lane 3*, 0.15 M KOAc; *lane 4*, 0.1 M KOAc; *lane 5*, 0.05 M KOAc. **C. Gel electrophoresis of the RNA removal method with different volumes of the 88% isopropanol/0.1 M KOAc solution on a 1.4% gel.** *Lane 1*¸ 2-log ladder; *lane 2*, control DNA subjected to RNase A only; *lane 3*, 1.2 volumes; *lane 4*, 0.6 volumes; *lane 5*, 0.5 volumes; *lane 6*, 0.4 volumes.

Based on all of these experiments attempting to remove the contaminating RNA from yeast chromosomal DNA, a modified method was developed. Figure 29 shows the overall analysis from the progression of these experiments. Lane 2 represents

chromosomal DNA preps digested with RNase A, without the pre-heating process, and the gel image indicated the presence of resistant RNA at the bottom. Lane 3 shows the chromosomal DNA prep that was incubated for 5 minutes at 65 °C, followed by digestion with RNase Cocktail and RNase V1 for 30 minutes at 37 °C. The gel image indicated that the RNA was significantly degraded into smaller fragments. Lane 4 represents the standard RNA/protein removal method described by Feliciello and Chinali applied to the DNA in lane 2 and the gel images showed that most of the small RNA fragments were removed but some of the higher molecular weight RNA molecules remained. Lane 5 represents the final optimized RNA removal protocol developed during the course of this project. First, chromosomal DNA was incubated for 5 minutes at 65 °C, followed by immediate digestion with RNase Cocktail and RNase V1 for 30 minutes at 37 °C. After digestion, 0.6 volumes of an 88% isopropanol/0.1 M KOAc solution was added. Following a 10 minute incubation at room temperature, the samples were centrifuged at 16,000 x g for 2 minutes and the supernatant was removed. The pellet was then washed with cold 70% ethanol, followed by resuspension in ddH_2O using the starting volume. Results shown in lane 5 showed that all detectable RNA was removed with no apparent chromosomal DNA loss.

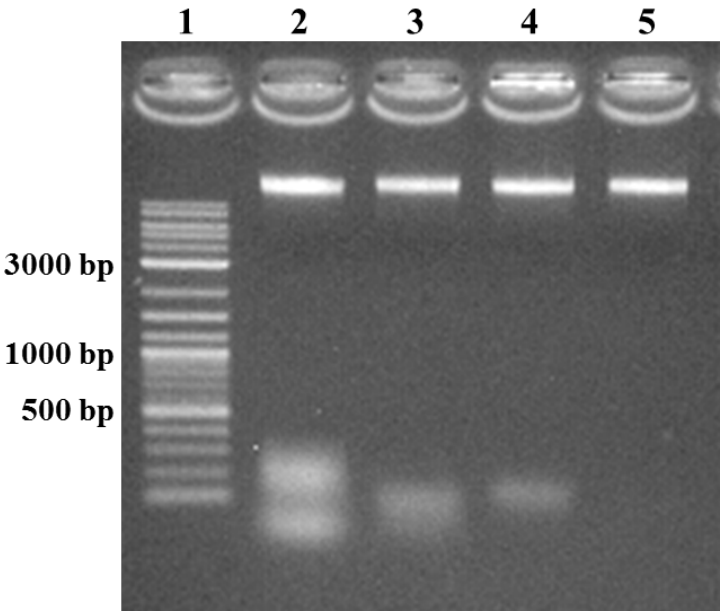

Figure 29. Demonstration of the overall progression of the RNA removal experiments on a 1.0% agarose gel. *Lane 1*, 2-log ladder; *lane 2*, DNA solution digested with RNase A without pre-heating; *lane 3*, DNA solution digested with RNase Cocktail and RNase V1 after heating; *lane 4*, standard RNA removal method; *lane 5*, modified RNA removal method.

Summary and Conclusions

The Ku complex is a vital component for the repair of broken chromosomes and maintaining the stability of telomeres. Cells lacking Ku display a temperature sensitivity phenotype when grown at elevated temperatures due to a destabilized telomere cap structure and increased nuclease degradation of the chromosome ends. This project attempted to test the rescue ability of new genes identified by a previous student from a cDNA plasmid screen that rescued the killing of *yku70* mutants even though 3 different strains and growth parameters were tested. However, overexpression of these genes did not show any rescue effect in *yku70* mutants.

Overexpression of telomerase Est2 polymerase and *TLC1* RNA subunits in *yku70* mutants has been reported by this lab and other labs to rescue the cell killing at 37 °C. This lab also showed that overexpression of Est2 polymerase without polymerase activity (*EST2-D530A* and *EST2-D670*) could rescue the mutants, indicating that the mechanism of rescue does not involve lengthening the telomeres. Furthermore, another lab previously reported that overexpression of another subunit, Est1, could rescue the cells at elevated growth temperatures (73). The plasmid encoding this gene was received by the Lewis lab; however, the rescue effect in *yku70* mutants could not be reproduced. Another lab published data where *EST1* overexpression could not alleviate the temperature sensitivity phenotype in *yku70* mutants unless fused to the telomere-binding protein Cdc13 (76). To further investigate the conflicting data regarding *EST1* overexpression, a plasmid was created in this project encoding *EST1* under the control of a strong constitutive promoter. The sequence of the coding region from start to stop codon was confirmed via DNA sequencing among other confirmation procedures. Again, the overexpression of Est1 in *yku70* mutants did not rescue cell killing at elevated growth temperatures. To finish testing the overexpression of telomerase subunits in *yku70* mutants, a plasmid encoding *EST3* was created as for *EST1* and the sequence of the coding region was confirmed via DNA sequencing. The overexpression of *EST3* in *yku70* cells also did not rescue cell killing at 37 °C.

Also investigated was another telomere associated protein, Pif1, and the DNA repair proteins, Sae2 and Srs2. Double mutants of *yku70 pif1*, *yku70 sae2*, and *yku70 srs2* were created and tested for the temperature sensitivity phenotype. *sae2* and *srs2* mutations did not affect the survival of the *yku70* cells. Survival of the *yku70 pif1* cells

was slightly increased when grown at 37 °C, which is likely due to the fact that Pif1 inhibition of telomerase is alleviated in the mutants. Also investigated was the ability of overexpressing Est2 polymerase and *TLC1* RNA in the *yku70 sae2*, *yku70 srs2*, and *yku70 pif1* cells to rescue growth at elevated growth temperatures. Both telomerase subunits were able to rescue *yku70* mutants in the absence of Sae2, Srs2, or Pif1, suggesting that their enzymatic activities are not required for telomere stabilization by Est2 or *TLC1* RNA.

During the course of this project it was necessary to purify DNA out of many different yeast strains, including wildtype BY4742 cells and several *yku70* mutant strains. Purifying DNA from yeast cells resulted in co-purification of large quantities of contaminating RNA. A portion of the RNA was resistant to standard digestion using RNase A, which produced 2 bands at the bottom of an agarose gel following electrophoresis. Qiagen anion exchange and Chroma Spin-1000 spin columns were then employed to purify the chromosomal DNA from the RNA; however, these columns were unsuccessful at removing the higher molecular weight RNA and resulted in DNA loss. The next step was to find a way to further degrade the larger RNA fragments into smaller oligomers. RNase I_f was used as an alternative to RNase A, but this enzyme resulted in non-specific degradation of chromosomal DNA in addition to the RNA. It was later discovered from this project that the addition of heat to the chromosomal DNA samples prior to RNase A digestion resulted in more efficiently degraded RNA. Then, a combination of the double-stranded RNA specific RNase V1 and RNase Cocktail (mixture of RNase A and RNase T1), in addition to heating the samples, resulted in the

most efficient method to degrade RNA contaminating chromosomal DNA preps. However, this approach did not digest all of the resistant RNA.

After discovering a method to degrade the higher molecular weight RNA more efficiently without depleting or degrading the chromosomal DNA, there was still the problem of purifying the chromosomal DNA away from the contaminating RNA. The alcohol precipitation method for RNA/protein removal method described by Feliciello and Chinali was employed; however, the published protocol did not remove all of the RNA. Thus, modifications were made to the protocol in order to optimize the removal of RNA while maintaining the DNA concentration. The modifications tested included centrifugation time, KOAc concentration, and volumes of isopropanol/KOAc added to the DNA/RNA solution. A new protocol was devised that efficiently removed the RNA to levels that could not be detected.

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