DNA DAMAGE RESISTANCE DETERMINED BY INTERPLAY BETWEEN TELOMERASE AND DNA REPAIR COMPLEXES

THESIS

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TABLE OF CONTENTS

LIST OF TABLES

Page

TABLES

LIST OF FIGURES

PAGE

FIGURE

CHAPTER 1

INTRODUCTION

Inside the nucleus, the blueprints for eukaryotic cells exist as tightly packed bundles of DNA molecules called chromosomes. The presence of highly reactive chemical groups causes these molecules to be extremely susceptible to damage. These groups include carbon-carbon double bonds, carbonyl groups, amine groups and phosphodiester bonds. Damage can occur through various processes. These processes include both endogenous and exogenous factors. Endogenous factors include oxidation and endonucleolytic cleavage. Exogenous factors include radiation and chemical agents (e.g. methyl methanesulfonate). Although these factors can lead to numerous types of damage, one of the more problematic types is a break within the DNA strands, either a double (DSB) or single-strand break (SSB). Furthermore, double-strand breaks (DSBs) are the more deleterious of the two. Cells that leave these breaks unfixed have to deal with several dire consequences. These consequences include loss of genomic information, induction of translocations, inaccurate repair leading to the formation of mutations, and/or apoptosis. In some cases, if the breaks are not repaired or are

inaccurately repaired, this may lead to cancer development. Because disrepair has several serious consequences, eukaryotic cells have been equipped with various methods for dealing with this situation. Two of the most prevalent methods the cell employs to repair DSBs are the homologous recombination and nonhomologous end-joining (NHEJ) pathways.

The yeast *Saccharomyces cerevisiae* (budding yeast) has become the model organism of choice for study of these two pathways. Although each pathway has been studied extensively in *S. cerevisiae*, there is still much to be discovered. *S. cerevisiae* presents an exceptional vessel for DSB repair study for several different reasons. These include its rapid generation time and well characterized genetic system. These factors have allowed for the characterization of several disease genes associated with radiation damage (1). Yeast also offer high DNA transformation efficiencies and with improved protocols, transformations have been simplified and are more efficient. Moreover, techniques such as *in vivo* oligonucleotide-mediated mutagenesis has allowed for efficient generation of site-specific mutations (2). These characteristics of yeast as a model genetic system have led to a greater understanding and elucidation of many cellular processes. Furthermore, genes identified in yeast frequently are found to have conserved homologs involved in these processes in more complex eukaryotes, such as humans (3).

Both homologous recombination and NHEJ utilize several protein complexes, one of which is involved in both processes. Protein complexes unique to NHEJ are Yku70/Yku80, Sir2/Sir3/Sir4, and Dnl4/Lif1/Nej1. Proteins unique to recombination are Rad51/Rad52/Rad54/Rad55/Rad57 and possibly some less understood proteins

(Figure 1). The complex active in both processes is the RMX complex, which consists of Rad50, Mre11 and Xrs2.

Each protein in RMX maintains a unique role within the function of the complex. A recent study has suggested that each protein within the RMX complex may come

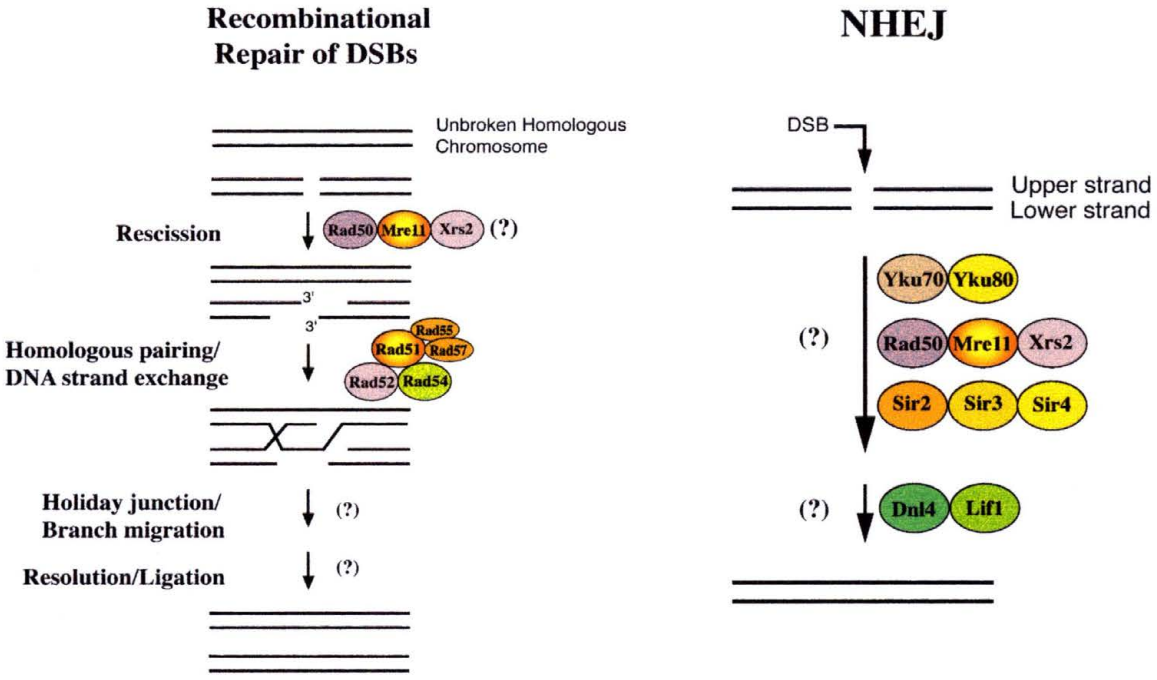
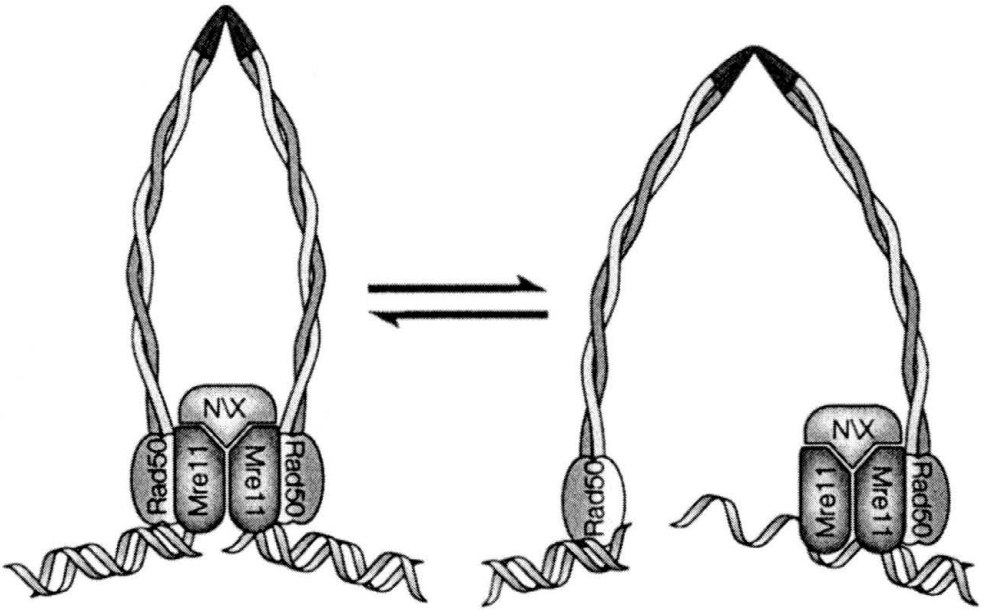


Figure 1. Representation of two DNA repair pathways and the proteins involved.

together and adopt a "headphone" shaped configuration (Figure 2) (4). Once in this configuration, each subunit employs a very different activity. Rad50 is a Zn^{2+} and Mg^{2+}-dependent protein which may act in recruiting the complex to broken DNA ends in the presence of ATP (5). Furthermore, the coiled-coil domain of Rad50 acts to hold the two ends of a DSB in close proximity and may also associate with a second Rad50 molecule to link sister chromatids in recombination (6). Mre11 is a manganese-dependent 3'-5' dsDNA exonuclease and ssDNA endouclease which is employed in the formation of 3'

ssDNA overhangs (Figure 3). This protein contains several highly conserved phosphoesterase motifs along with two DNA binding domains (Figure 4) (7, 8). Studies have also shown that Mre11 is directly bound to Rad50 forming the core within the complex (4). Another striking indication of the importance of Rad50 and Mre11 is the



RMX bound to broken DNA ends

Resection of DNA to form 3' tail

Figure 2. Proposed "Headphone" configuration of RMX bound to broken DNA ends.

presence of identifiable homologs through a wide range of species, including humans, and their function in the detection of DSBs and cell cycle control (9, 10).

Mutations in any of the three RMX proteins cause cells to become highly sensitive to ionizing radiation, DNA damage-inducing chemicals and endonucleases. Also, the mutant cells exhibit genomic instability such as chromosome loss and telomere shortening (11). Furthermore, inactivation of the complex (hRad50/hMre11/Nbs1)

within human cells results in similar deficiencies and has been linked to the disorders Nijmegen Breakage Syndrome and Ataxia Telangiectasia-like Disorder, both of which are characterized by a predisposition to cancer formation and hypersensitivity to ionizing radiation (12).

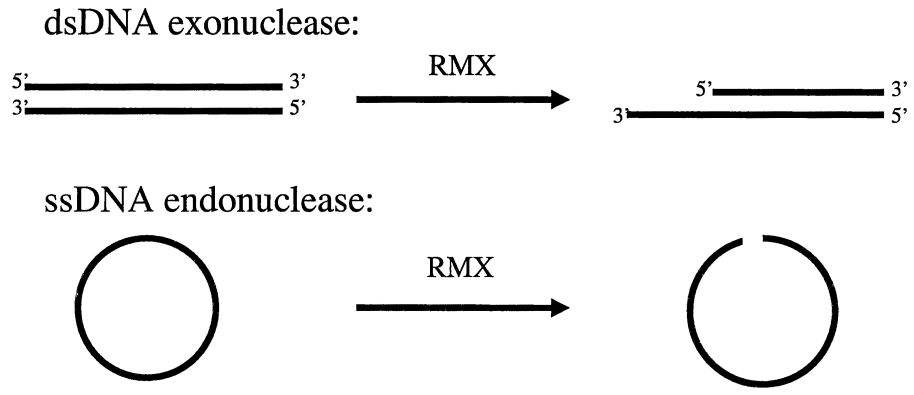


Figure 3. Nuclease activities of the RMX complex

Eukaryotic chromosomes contain specialized structures that protect their ends. These structures are referred to as telomeres and are composed of several repeated DNA sequences. These sequences are regions of association for various DNA-binding proteins (13). Telomeres are necessary to preserve genomic integrity and prevent premature cell death. However, one cellular shortcoming, because of the nature of replication, is the short stretch of DNA which is lost after each round during S-phase of the cell cycle. Therefore, cells possess the telomere regenerating protein complex, telomerase (14).

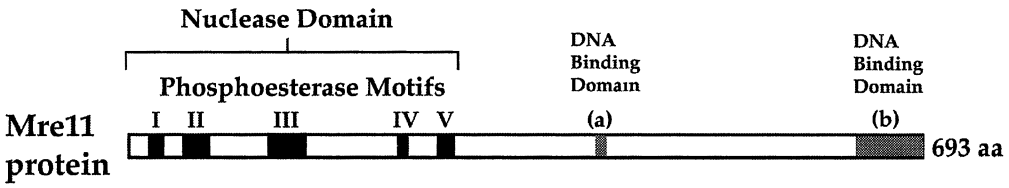


Figure 4. Schematic of Mre11 with the phosphoesterase motifs labeled as I-IV, and DNA binding sites (A and B).

Telomerase is inactive within most mammalian tissues; however, it remains active in rapidly dividing cells and germ cells where genomic integrity is crucial. The inactivity of telomerase in somatic cells leads to a progressive shortening of chromosomal ends. This time-dependent shortening has suggested ties to aging and age-related disease (15). Furthermore, telomere reactivation is considered to be a pivotal step in carcinogenesis (16). Since nearly 90% of all cancers have telomerase activity reactivated, this situation offers insight into the immortality of cancerous cells. Therefore, current research has been focused on understanding the relationship between telomerase, cellular immortality and development of therapeutics for confronting tumor production. Furthermore, as mentioned previously, telomere shortening has been associated with aging, which has increased research interest in this area as well.

In yeast, telomerase consists of four protein subunits, an RNA subunit and possibly several additional less characterized proteins. The more understood subunits include the Est1, Est2, Est3 and Cdc13 proteins and *TLC1* RNA. Est2 is the catalytic subunit employing an RNA-dependent DNA polymerase activity. Est1 and Cdc13 have been shown to possess DNA binding activity with specificity directed towards 3' tails.

The function of Est3 remains unknown (13). *TLC1* is a 1301 bp RNA that contains a 17 nucleotide long sequence employed as a template for replication elongation at the telomeres. As depicted in Figure 5, during S-phase of each cell cycle *TLC1* RNA anneals to 3' overhangs at the end of each chromosome, and complementary DNA is synthesized by Est2. .

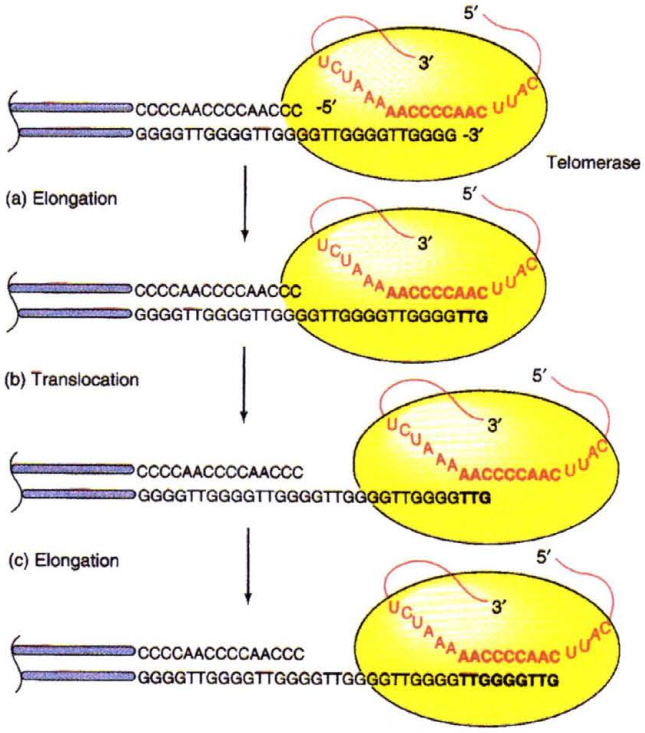


Figure 5. Schematic of telomerase RNA-mediated telomere replication.

The absence of *TLC1* RNA or other telomerase subunit results in cellular senescence and telomere shortening. *TLC1* was first isolated from a screen for genes which, when overexpressed, would suppress gene silencing at the telomeres (17). This result presented evidence for the telomeric association of *TLC1* RNA. Furthermore, *tlc1* strains displayed progressively shorter telomeres when compared to wild-type strains

(17). The *TLC1* RNA contains two very important regions with respect to telomere addition and possibly protein interactions with other telomerase components. The first region is one known as the "stem loop" region. This region in the 5' end of the RNA consists of approximately 48 nt and is necessary for the disruption of silencing at the telomeres (18). The second is termed the template region, located at nt 468-484. This region consists of 17 nt, is composed of CACA repeats (5'-CACCACACCCACACACA-3') and is the region of the RNA used as a template for addition of repeats to telomere ends. Both of these distinct regions comprise a small part of the complete 1301 nt *TLC1* RNA. In addition, recent studies demonstrate that *TLC1* RNA contains binding sites for several proteins, including Est1, Est2 and theYKu70/YKu80 complex (18, 19, 20). Besides its crucial implication in the maintenance of telomeric ends, previous studies in this lab involving *TLC1* have exposed a distinct role in DNA repair (21).

As stated previously, cells possessing mutations in the genes coding for the RMX proteins display increased sensitivity to radiation and damage-inducing chemicals and an increase in chromosome rearrangements and loss (11, 22). However, previous studies in this lab have suggested that these conditions can be alleviated by elevated expression of two genes, *EXO1* and *TLC1* (21). *EXO1*, which encodes a 5'-3' exonuclease, was the first gene analyzed. Results indicated that Exo1 could substitute for the RMX complex in the formation of the 3' overhang which occurs during the first step and is necessary for the initiation of repair through the recombination pathway (21). Moreover, no significant effects of *EXO1* on repair through NHEJ could be detected.

The mechanism through which the second, and more surprising gene *TLC1* rescues DNA repair in RMX mutants remains unclear. Several mechanisms have been

proposed as an answer to this question. First, suppression of lethality may be the result of *de novo* telomere addition to each end of the DSBs. Second, suppression may proceed through elevated levels of repair through one or both of the pathways, recombination and NHEJ. These latter effects may involve a titration event through which *TLC1* RNA may act as a recruiting agent. More specifically, *TLC1* RNA may attract telomerase-associated end binding proteins and thereby prevent their association with the ends involved in a DSB, thus, allowing repair complexes greater access to the break.

The primary goals of the current project were to investigate the roles of the two known functional domains of the RNA (the stem loop and template region) and to elucidate the mechanism of *TLC1*-mediated repair. This was accomplished through the determination of which DSB repair pathway(s) are involved in *TLC1*-mediated DNA repair. In addition, we investigated the effects of certain *TLC1* derivatives on repair and assessed the importance of the stem loop and template regions of *TLC1* RNA.

CHAPTER 2

MATERIALS AND METHODS

I. MATERIALS

General Reagents

Ammonium sulfate (granular), sodium chloride and sodium dodecyl sulfate (SDS) were purchased from Mallinckrodt AR (Paris, Kentucky). Agarose and ethidium bromide were purchased from Shelton Scientific, Incorporated (Shelton, CT). Methyl methanesulfonate (MMS) was obtained from Fluka. Lithium acetate dehydrate, calcium chloride, glycerol, polyethylene glycol (PEG-4000), Sarkosyl (N-lauroyl-sarcosine), Tween 20 and magnesium chloride were purchased from Sigma Chemical Company (St. Louis, MO). Tris base was purchased from Invitrogen Life Technologies (Carlsbad, CA).

Bacteriological and yeast media

All amino acids, plate agar, D-(+)-glucose, ampicillin, and D-galactose were purchased from Sigma Chemical Co. (St. Louis, MO). Difco bacto peptone, bacto yeast extract, bacto tryptone and bacto yeast nitrogen base were purchased from Becton Dickinson Microbiological Systems (Sparks, MD).

Enzymes and PCR reagents

Restriction enzymes were purchased from New England Biolabs (Beverly, MA). Taq Plus Long and PCR reagents were purchased from Stratagene (La Jolla, CA).

Cell culture solutions and media

For general, non-selective growth, yeast cells were grown on YPD (rich) media (1% bacto yeast extract, 2% bacto peptone, 2% glucose, 2% bacto agar). In order to assess mitochondrial function, yeast cells were grown on YPG (1% bacto yeast extract, 2% bacto peptone, 2% bacto agar, 3% glycerol). For plasmid selection, yeast cells were grown on synthetic media with drop-out mix (0.17% yeast nitrogen base without amino acids or ammonium sulfate, 0.5% ammonium sulfate, 2% glucose, 2% bacto agar, plus all essential amino acids minus amino acids used for selection). Methyl methanesulfonate (MMS) plates were made using synthetic media or YPD plus MMS mixed to obtain various concentrations of the DNA methylating agent.

Yeast strains and plasmids

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

Table 1 *S cerevisiae* strains

Strain	Genotype	Reference/Source
T334	*MATα ura3-52 leu2-3,112 Δtrp1..hisG reg1-501 gal1 pep4-3 prb1-1122*	(21)
YLKL350	T334, *Δhis3 :[GAL1·:EcoRI TRP1]*	(21)
VL6α	*MAT α ura3-52 trp1(Δ63) lys2-801 his3- Δ200 met14 ade2-101*	LARIONOV *et al* (1994)
VL6-48	VL6α, *Δleu2::G418^r*	LAB STRAIN
YLKL276	VL6α, *Δrad52.·hisG*	(21)
YLKL398	YLKL350, *Δrad50.:G418^r*	LAB STRAIN
YLKL503	VL6α, *Δmre11.·G418^r*	(21)
YLKL544	VL6α, *Δdnl4.:G418^r*	LAB STRAIN
YLKL549	VL6α, *Δmre11 ade2-TRP1-ade2*	LAB STRAIN
YLKL593	VL6-48, *Δyku70 ·HIS3*	LAB STRAIN
YLKL613	YLKL544, *Δmre11 :HygB*	LAB STRAIN
YLKL615	YLKL276, *Δmre11::G418^r*	LAB STRAIN
YLKL641	VL6α, *Δmre11-D16A*	LAB STRAIN
YLKL724	YLKL641, *exo1 URA3*	LAB STRAIN
YLKL725	YLKL503, *exo1 URA3*	LAB STRAIN
YLKL783	YLKL549, *ADE2 Trp1*	LAB STRAIN

Table 2 Plasmids

Plasmid	Description	References/Source
pCDNA50 3	*CEN/ARS URA3 GAL1p ·TLC1*	(21)
pRS424	*2 μ TRP1*	SIKORSKI AND HIETER (1989)
pRS314	*CEN/ARS TRP1*	SIKORSKI AND HIETER (1989)
pRS315URA3	*CEN/ARS URA3 LEU2*	This Lab
pLKL64Y	*2 μ LEU2 ADH1p· TLC1*	This Lab
pTRP61	*2 μ TRP1 GAL1p::TLC1*	(17)
pLKL74Y	*2 μ TRP1 GAL1p::TLC1*	This Work
pLKL75Y	pLKL74Y, Δ(*Ecl136II-AflII*)	This Work
pLKL76Y	pLKL74Y, Δ(*Ecl136II-HpaI*)	This Work
pLKL77Y	pLKL74Y, Δ(*Ecl136II-NcoI*)	This Work
pLKL78Y	pLKL74Y, Δ(*BamHI-StuI*)	This Work
pLKL79Y	pLKL74Y, Δ(*BamHI-NcoI*)	This Work
pTCG 3X Stem	*2 μ TRP1 GAL1p··TLC1(3xSTEM)*	(18)
pTCGΔCA	*2 μ TRP1 GAL1p: TLC1-ΔCA*	Gift from Dan Gottschling

II. METHODS

Chromosomal and plasmid DNA purification

For chromosomal DNA, a MasterPure™ Purification Kit by Epicentre Technologies was used following the kit protocol. Plasmid DNA was purified using a rapid boiling lysis method (23).

Yeast transformations

Yeast transformations were performed using either a high efficiency method described by Gietz *et al.* (24) or a rapid DMSO-based transformation method by Soni *et al.* (25).

Dilution pronging survival assays

In a sterile 96-well microtiter dish, yeast cells were inoculated in selective liquid media (total volume ~220 µl per well) and grown overnight at 30 °C. Next, a series of 5-fold dilutions of the overnight culture were made along the length of the dish. The cells were then pronged onto control plates that were selective for the plasmid and also onto the selective plates containing varying concentrations of DNA damaging agents (e.g. MMS) or media containing 0.5% galactose for the induction of *Eco*RI endonuclease. Strains used for the *Eco*RI expression studies were derivatives of YLKL350, which contains a *GAL1p::EcoRI* cassette integrated into the *HIS3* locus on chromosome XV. The plates were analyzed after 3-4 days growth at 30° C for sensitivity to induced double strand breaks.

Removal of TRP1 from YLKL549 (T334-Δmre11:: ade2-TRP1-ade2)

YLKL549 cells, which have the *ADE2* gene disrupted with *TRP1* (*ade2-TRP1-ade2*), were placed in 500 µl of YPDA broth in a 1.5 ml microfuge tube. These cells

were subsequently spread onto synthetic Glu-Ade plates to select for cells which had recombined the *ade2* genes and become Ade^+. Five to ten colonies were patch-purified onto -Ade, -Trp and YPG plates. Trp^- and YPG^+ (mitochondria proficient) patches were used for subsequent experiments.

Production of the pLKL74Y (2μ GAL1p::TLC1 TRP1) plasmid

Restriction digest of pCDNA50.6 and pRS424. pCDNA50.6 (*CEN/ARS GAL1p::TLC1 URA3*) (21) was digested using *Eco*RI and *Not*I to remove the *GAL1p::TLC1* cassette in the following reaction. In a 1.5 ml microfuge tube the following were added: 10 μl pCDNA50.6, 64 μl ddH_20, 20 μl KGB buffer, 3 μl *Eco*RI and 3 μl *Not*I. The mixture was incubated at 37° C for 3-4 hours. After incubation, the mixture was placed in 65-75° C for enzyme inactivation. A sample of the mixture was run on a 0.7% preparative agarose gel for purification of the *GAL1p::TLC1* fragment.

pRS424 was digested with *Eco*RI and *Not*I, to create a large vector piece, in the following reaction. In a 1.5 ml microfuge tube, the following were added: 8 μl pRS424, 66 μl ddH_2O, 20 μl 5xKGB buffer, 3 μl *Eco*RI and 3 μl *Not*I. The mixture was incubated at 37° C for 3-4 hours. After incubation, the mixture was treated with shrimp alkaline phosphotase to prevent re-ligation of the vector fragment in the following reaction: 14 μl ddH_2O, 4 μl 5xKGB buffer and 2 μl shrimp alkaline phosphatase. The mixture was incubated at 37° C for 1 hour then placed at 65-75° C for 15 min to deactivate the enzymes. A sample was run on the aforementioned 0.7% preparative gel for purification of the large vector fragment.

Gel purification of GAL1p::TLC1 and pRS424 vector fragments. Samples of each digest were run on 0.7% agarose gel in 1XTBE. The gel was stained in an ethidium

bromide solution and destained using ddH_2O. After destaining, the gel was briefly visualized using a UV light source. Both the large vector fragment and *GAL1p::TLC1* fragment were removed from the gel using Millipore tweezers. The gel plug was placed into a 1.5 ml microfuge tube containing a glass wool packed pipette tip for DNA extraction. The tube was spun in a microfuge at 8,000 x g for approximately 1 minute. The glass wool containing pipette tip was discarded and the tube was respun for 2 minutes at full speed. The supernatant was transferred to a new microfuge tube and precipitated.

Ligation of GAL1p::TLC1 fragment and pRS424 vector fragment. Quantification of each fragment was done with 2 µl sample on a Hoefer fluorometer (Hoefer Pharmacia Biotech Inc., California). The two fragments were mixed and co-precipitated at a 1:5 molar ratio (vector:insert) in the following reaction mixture: 7 µl (4 ng/ml) pRS424 vector fragment, 8 µl (7 ng/ml) *GAL1p::TLC1* fragment, 5 µl ddH_2O, 2 µl 3M NaOAc, 55 µl 100% EtOH. The mixture was microfuged at full speed for 15 min, gently washed with 70% EtOH, respun for 3 min. The supernatant was transferred, the pellet was desiccated using a Savant Speedvac SC110 (Savant, New York) and resuspended in the following ligation mixture: 9.8 µl ddH_2O, 1.2 µl 10x T4 ligase buffer, 1 µl T4 DNA ligase. The reaction mixture was incubated at 15° C overnight, transformed into DH5α cells, and DNA minipreps performed on the resulting colonies for LB + Amp plates. Plasmids containing the correct insertion were designated pLKL74Y.

Creation of GAL1p::TLC1 deletion derivatives from pLKL74Y. pLKL74Y was digested to yield five different *TLC1* derivatives. Each reaction mixture was as follows: 4 µl pLKL74Y, 10 µl 5xKGB buffer, 34 µl ddH_20, 0.9 µl enzyme 1, 0.9 µl enzyme 2.

Each mixture was incubated at 37° C for 2-4 hours. The enzymes used for each reaction were: pLKL75Y- *Ecl136*II/*Afl*II, pLKL76Y- *Ecl136*II/*Hpa*I, pLKL77Y- *Ecl136*II/*Nco*I, pLKL78Y- *Bam*HI/*Stu*I, pLKL79Y- *Bam*HI/*Nco*I. A fill-in reaction was performed for pLKL75Y, 77Y, 78Y and 79Y. After incubation, the following was added to each of the mentioned reaction mixtures: 18 µl ddH_2O, 5 µl 5xKGB buffer, 1 µl 2.5 mM dNTP's, 1 µl T4 DNA polymerase. Each mixture was incubated at 12-15° C for 20 min followed by 75° C for 10 min to inactivate the polymerase enzyme.

Transformation of DH5α with pLKL74Y and derivatives. All DNA ligation mixtures were transformed into *E. coli* DH5α cells separately using the following method: into a Falcon 2059 tube, 100µl of cold KCM buffer, 1-15 µl of the respective plasmid DNA, and 100 µl of thawed competent DH5α cells were added. The mixture was placed on ice for 10-20 minutes and then immersed at ~25-30° C for 10 min. 0.9 ml of SOC broth was added and each tube was shaken at 37° C for 40-60 min. Aliquots of 20 µl and 300 µl were spread to LB-plus-ampicillin plates.

CHAPTER 3

RESULTS AND DISCUSSION

This research project focused on the mechanism by which altering cell levels of the telomerase RNA component, *TLC1*, can rescue the DNA repair defects of cells lacking the Rad50:Mre11:Xrs2 (RMX) complex. Cells lacking RMX possess many phenotypes including increased sensitivity to ionizing radiation (X-rays) and DNA damage inducing chemicals (MMS, bleomycin, etc.), genomic instability and telomere shortening. Earlier work indicated that overexpression of *TLC1* RNA could alleviate the hypersensitivity of RMX mutants to MMS and radiation (21). The RMX complex is utilized in both NHEJ and recombination repair; therefore, the specific pathway involved in rescue of RMX mutants by *TLC1* RNA overexpression was one main concern. Furthermore, elucidation of specific *TLC1* RNA regions absolutely necessary for RMX-mediated double-strand break (DSB) repair, as well as the rescue of other DNA repair deficient mutants (*yku70*), was a second important question. A third set of experiments addressed the possibility of *EXO1* acting as a back-up nuclease in *mre11* mutants.

TLC1 expression increases repair of chromosomal DSBs

MMS indirectly leads to DSBs through methylation of nitrogen groups present within purine bases. The methylation exists as 7MeG (80-85%), 3MeA (9-12%), 3MeG (0.3-0.7%), O^6MeG (0.3%), 7-methyladenine (1.8%), as well as several minor lesions. Although there are several different lesions caused by methylation, the most detrimental effects have been suggested to come from 3MeA and O^6MeG (26). These lesions are thought to lead to DSBs through inhibition of DNA replication (21). Because MMS causes several different modifications, it was necessary to establish whether *TLC1* RNA specifically enhances the repair of DSBs.

*Eco*RI is a restriction endonuclease that cleaves at a specific sequence (G^AATTC) of base pairs within DNA (Figure 6). Therefore, the sole lesion created through this cleavage is a DSB possessing 5' overhangs. To investigate the impact of *TLC1* on repair of DSBs, strain YLKL398 (*GAL1p::EcoRI, Δrad50*) with or without the *TLC1* plasmid pLKL64Y (*ADH1p::TLC1 LEU2*) was assayed for survival (Figure 7).

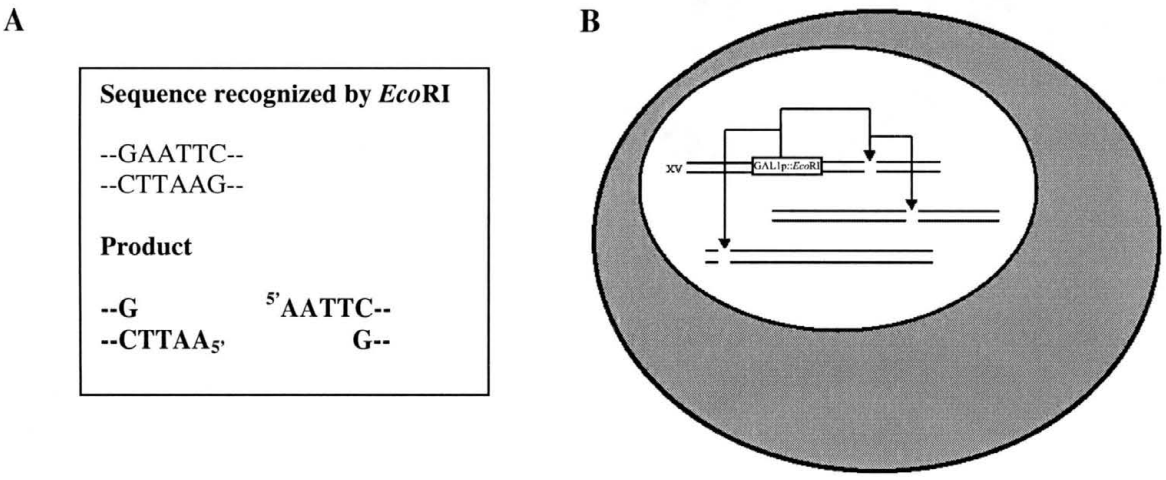


Figure 6. (A) Sequence recognition and product of *Eco*RI. (B) *In vivo* expression of *Eco*RI.

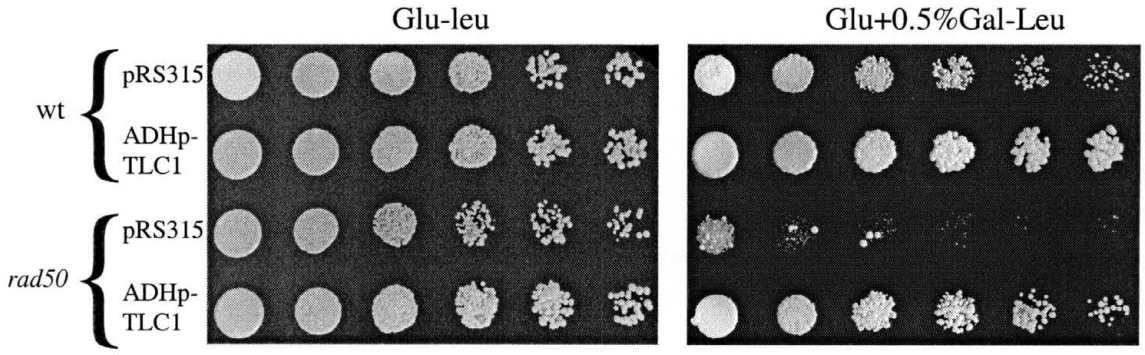


Figure 7. Expression of *TLC1* RNA suppressed *Eco*RI induced lethality in *rad50* mutants compared to wildtype. *rad50* mutants were plated to media containing 0.5% galactose for induction of *Eco*RI. wt =wildtype

As seen in Figure 7, when compared to mutants plated on glucose media (no *Eco*RI induction) expression of *Eco*RI caused increased lethality within *rad50* mutants containing only the cloning vector pRS315. In contrast, *rad50* mutants constitutively expressing *TLC1* RNA from the *ADH1* promoter exhibited increased survival in the presence of *Eco*RI induction. Interestingly, increased cellular levels of *TLC1* RNA increased the growth rate of wildtype cells ($RAD50^{+}$), seen as larger colonies in Figure 7, when *Eco*RI was expressed. These observations indicate that *TLC1* RNA is suppressing negative effects resulting from the presence of DSBs.

TLC1- mediated repair does not proceed through NHEJ

The nonhomologous end-joining repair pathway exists as a secondary pathway within *S. cerevisiae*. However, studies have suggested that NHEJ is crucial in repair of

specific types of DSBs and for telomere stabilization (27). Furthermore, NHEJ deficiencies have been implicated in several phenotypes such as decreased survival after induction of *Eco*RI and inability to recircularize cohesive-ended plasmids (Table 3).

To determine whether *TLC1* RNA-mediated repair proceeds though the nonhomologous end-joining pathway, MMS sensitivity was analyzed in *mre11 dnl4* mutants as well as an *mre11* control strain (Figure 8). *DNL4* encodes a subunit of the DNA ligase IV complex, which is essential for NHEJ. Inactivation of this gene should eliminate *TLC1*-mediated repair if it involves elevation of NHEJ. The analysis was performed through 5-fold dilution pronging of strains YLKL503 (*mre11*) and YLKL613 (*mre11 dnl4*) each containing the plasmids pRS314 or pTRP61 (*GAL1p::TLC1, TRP1*). The dilutions were plated onto media containing 3% galactose with either 0.10 or 0.35 mM MMS.

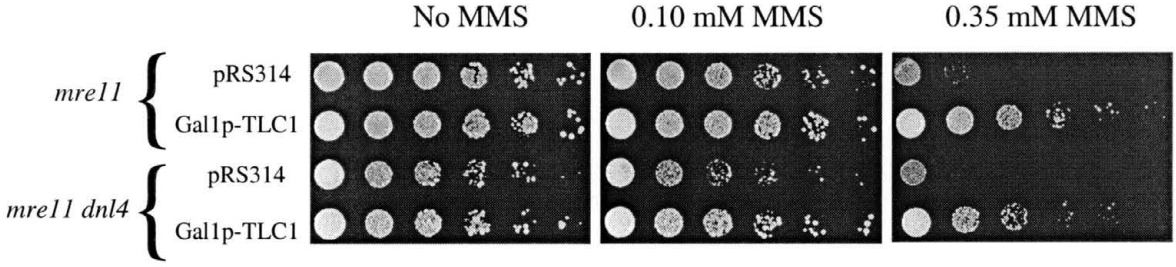


Figure 8. Overexpression of *TLC1* RNA suppressed MMS induced killing in *mre11 dnl4* (YLKL613) mutants

When expressing only the empty cloning vector (pRS314), *mre11 dnl4* mutants showed inhibition similar to *mre11* single mutants at both 0.10 mM and 0.35 mM MMS concentrations. However, overexpression of *TLC1* RNA alleviated this inhibition at both

Table 3. Phenotypes associated with NHEJ- and recombination-deficient strains of Saccharomyces cerevisiae[a]

	Rejoining of cohesive-ended plasmids DSBs	Survival after induction of EcoRI in vivo	Deletion formation in dicentric plasmids	Cell survival after treatment with		
				Gamma	Bleomycin	MMS
NHEJ-defective						
yku70	- - -	- - -	- - -	+	–/+	–/+
yku80	- - -	- - -	nd	+	–/+	–/+
dnl4	- - -	–	nd	+	+	+
lif1	- - -	nd	nd	+	nd	nd
sir2	- - -	- - -	- - -	+	+	–/+
sir3	- - -	- - -	- - -	+	+	–/+
sir4	- - -	- - -	- - -	+	+	–/+
Recombination- and NHEJ-defective						
rad52	- - -	- - -	- - -	- - -	- - -	- - -
mre11(rad58)	- - -	- - -	- - -	- - -	- - -	- - -
xrs2	- - -	- - -	- - -	- - -	- - -	- - -
Recombination-defective						
rad52	~ +	+	+	- - -	- - -	- - -

[a]Symbols: +, near wildtype; –, moderatley reduced relative to wildtype cells; - - -, severely defecient; nd, no data

MMS concentrations. The similar suppression of MMS-induced killing by *TLC1* RNA in both strains indicates that the nonhomologous end-joining repair pathway is not required for the enhanced repair. This result is consistent with the use of NHEJ in *S. cerevisiae* as a secondary means of DSB repair when compared to homologous recombination.

Homologous recombination is required for TLC1 RNA-mediated repair

The previous result involving NHEJ-deficient cells left open the possibility that *TLC1*-mediated DSB repair may involve increased homologous recombination. This suggestion is consistent with the idea that homologous recombination is the primary repair pathway utilized within *S. cerevisiae* and is highly efficient (24, 28). Homologous recombination involves genes present in the RAD52 epistasis group which include *RAD50-RAD59*, *MRE11* and *XRS2*. Deletion of any constituent of this epistatic group, with the exception of *RAD50/MRE11/XRS2* in which recombination is only partially deficient, leads to a strong reduction in recombination repair. Therefore, to investigate the role of homologous recombination in *TLC1* RNA suppression, our laboratory assayed survival in *mre11 rad52* cells after exposure to MMS. Again, this assay was performed by 5-fold dilution pronging of cells YLKL503 (*mre11*) and YLKL615 (*mre11 rad52*) containing the plasmids pRS314 or pTRP61 (Figure 9).

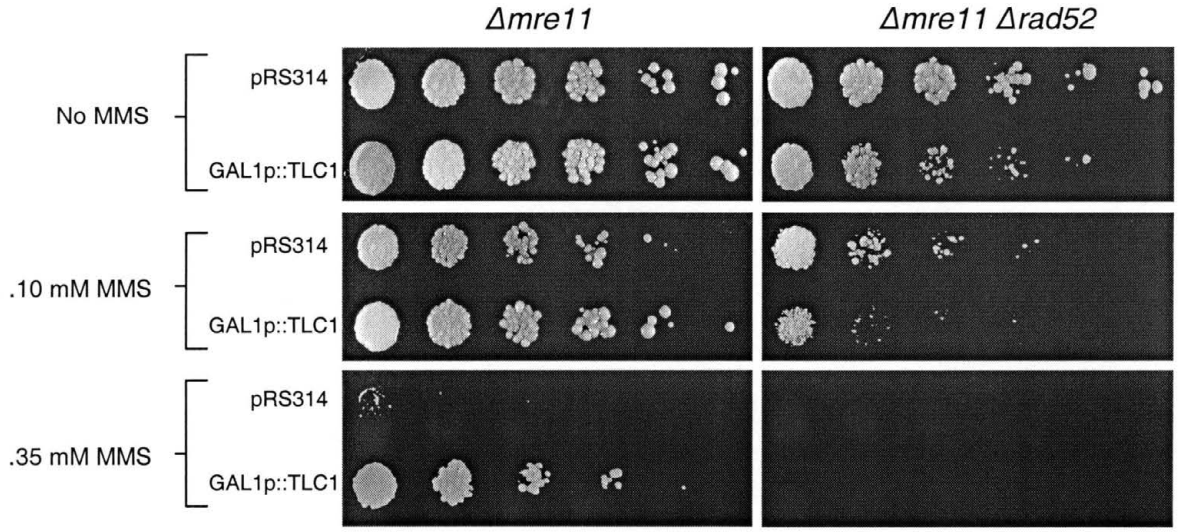


Figure 9. Overexpression of *TLC1* RNA did not suppress MMS induced killing of *mre11 rad52* (Rec$^-$) as compared to *mre11* single mutants.

The *mre11 rad52* mutants were extremely sensitive to 0.35 mM MMS and *TLC1* RNA overexpression did not exhibit the suppression of MMS-induced killing seen in *mre11* single mutants. This is an important observation which suggests that *TLC1* RNA suppression requires homologous recombination. Although the exact role *TLC1* RNA undertakes within recombination remains uncertain, one possible explanation may be a titration effect on the part of *TLC1* RNA by binding to proteins which have affinity for broken DNA ends. These proteins may inhibit repair complexes from accessing the DSB ends.

Exo1 provides a backup nuclease activity in the absence of Mre11

EXO1 encodes a 3'-5' exonuclease implicated in DNA replication, mismatch repair and homologous recombination. Previous studies conducted in our laboratory

revealed that overexpression of *EXO1* in RMX mutants leads to suppression of killing by MMS (and other DNA damaging agents) (21). This observation strongly suggests that in the absence of the RMX complex, Exo1 may perform, although inefficiently, the nuclease activity necessary in the initial step of homologous recombination (Figure 10). To investigate whether *EXO1* is acting as a secondary nuclease in RMX mutants and whether *TLC1* RNA overexpression suppresses MMS-induced killing in a strain lacking both nucleases, survival was monitored in *mre11 exo1::URA3* double mutants as compared to *mre11* single mutants when exposed to MMS (Figure 11).

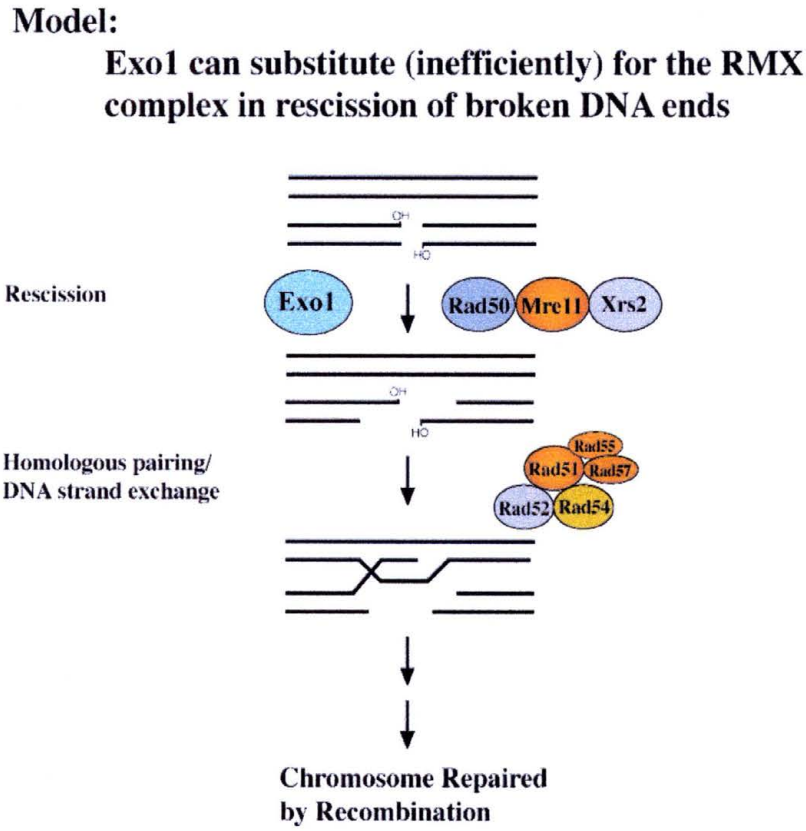


Figure 10. Proposed model indicating the involvement of Exo1 in the initial resection step of homologous recombination.

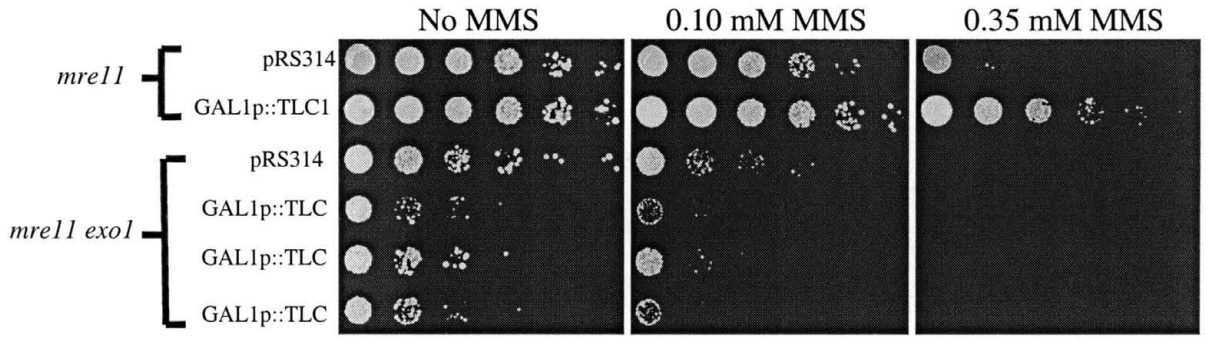


Figure 11. B.) Overexpression of *TLC1* did not suppress MMS-induced killing in *mre11 exo1::URA3* double mutants. Also, these double mutants show decreased survival on media without MMS. Strains YLKL503 (*mre11*) and YLKL725 (*mre11 exo1::URA3*) were plated on galactose media containing increasing MMS concentrations.

As seen in Figure 11, *TLC1*RNA overexpression did not alleviate the lethality in *mre11 exo1::URA3* double mutants though *mre11* single mutants were rescued. Furthermore, *mre11 exo1::URA3* mutants exhibited slower growth on galactose media which does not contain MMS. This observation not only reinforces the importance of the initial nuclease step in homologous recombination, but also reinforces the importance of *EXO1* as a backup nuclease after loss of Mre11 function. This result is consistent with previous findings implicating *EXO1* in the rescue of RMX mutants when exposed to DNA damage-inducing agents (21). These results allow us to propose a model for the role of *EXO1* interaction with *TLC1* in the absence of the RMX complex (Figure 12) (described in more detail below).

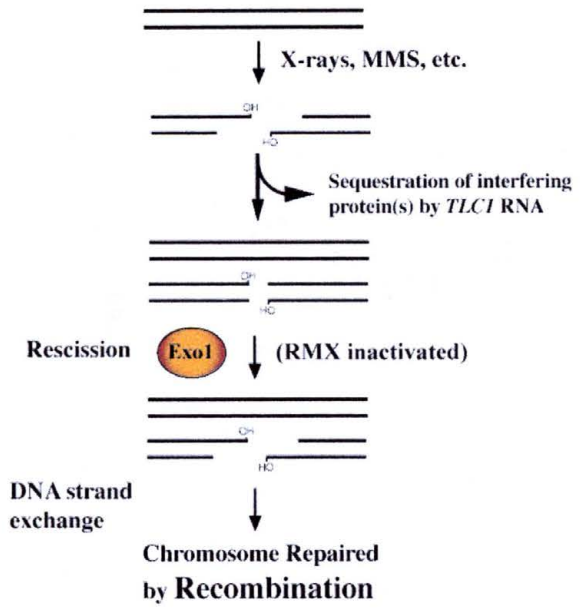


Figure 12. Possible model indicating *TLC1* RNA involvement in conjunction with Exo1 to repair DSBs in the absence of RMX

Analysis of suppression by TLC1 gene deletion mutants

Because previous studies in our laboratory have been conducted using the entire 1301bp *TLC1* gene, an interesting question that we decided to address was which regions of *TLC1* RNA are absolutely necessary for enhanced DSB repair. Answering this question may provide valuable information that could lead to an increased understanding of the interaction between RMX and *TLC1*, and perhaps, help increase our understanding of *TLC1*'s role in DSB repair. To address this question, our laboratory constructed or obtained from outside sources several *TLC1* RNA derivatives.

TLC1 deletions derived from pRS424 did not suppress MMS-induced killing

An initial set of experiments involving pRS314 (*CEN/ARS TRP1*) containing *TLC1* (pLKL70Y) failed because this construct did not exhibit strong enough suppression

of MMS-induced killing, though it did rescue killing of *yku70* mutants at elevated temperatures. Therefore, we decided to test deletions derived from the cloning vector pRS424. pRS424 is a multi-copy plasmid containing the *TRP1* marker (29). The use of this plasmid in our MMS survival assay will alleviate low yield problems (low concentration of *TLC1* RNA produced) experienced with the single copy centromeric plasmid pLKL70Y. A *TLC1* gene cassette, which was released from pCDNA50.3 (*GAL1p::TLC1 URA3*), was ligated into pRS424 as described in Methods. The resulting plasmid (*2μ GAL1p::TLC1 TRP1*) was named pLKL74Y. This plasmid was digested using several different restriction enzymes (Figure 13). Five distinct derivatives were produced from pLKL74Y (pLKL75-79Y). pLKL75-77Y had increasing amounts of the 3' end deleted.

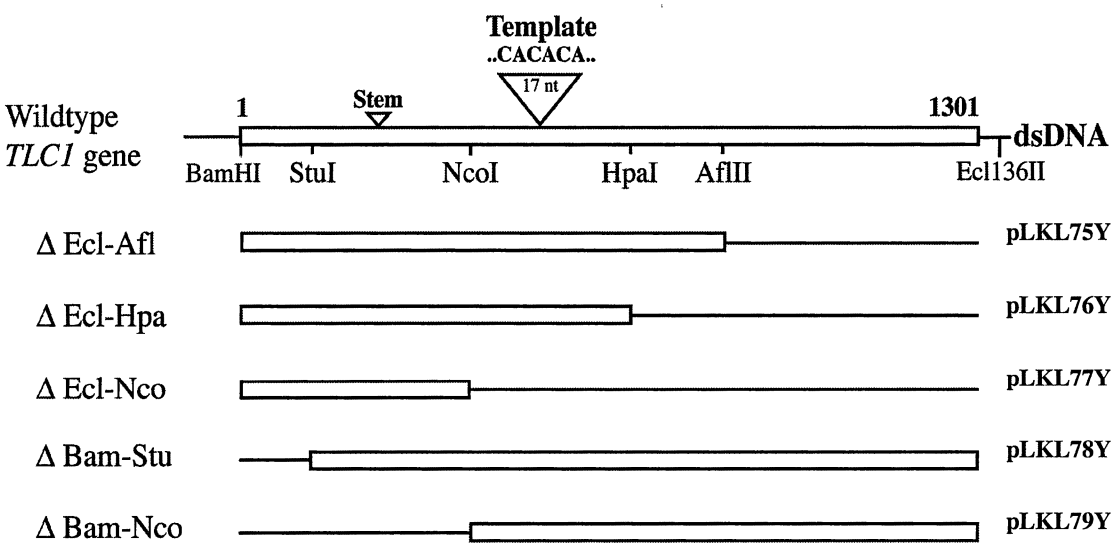


Figure 13. Schematic showing products formed by *TLC1* digestion. The appropriate enzymes are shown with their approximate locations.

while pLKL78Y and pLKL79Y had increasing amounts of the 5' end deleted, including the stem (Figure 13).

Each of the derivatives was transformed into the strains YLKL783 (*mre11*) and YLKL593 (*yku70*). *YKU70* and *YKU80* encode subunits of the Ku heterodimer which has been implicated in the binding of DNA ends and mediation of NHEJ (30). Studies have also linked Ku with telomeric DNA stability and protection, and mutations of *ku* cause elevated levels of telomere destabilization and cell death when placed at 37° C (31). Each strain was assayed for increased survival, YLKL783 (*mre11*) against MMS and YLKL593 (*yku70*) at 37° C (Figure 14).

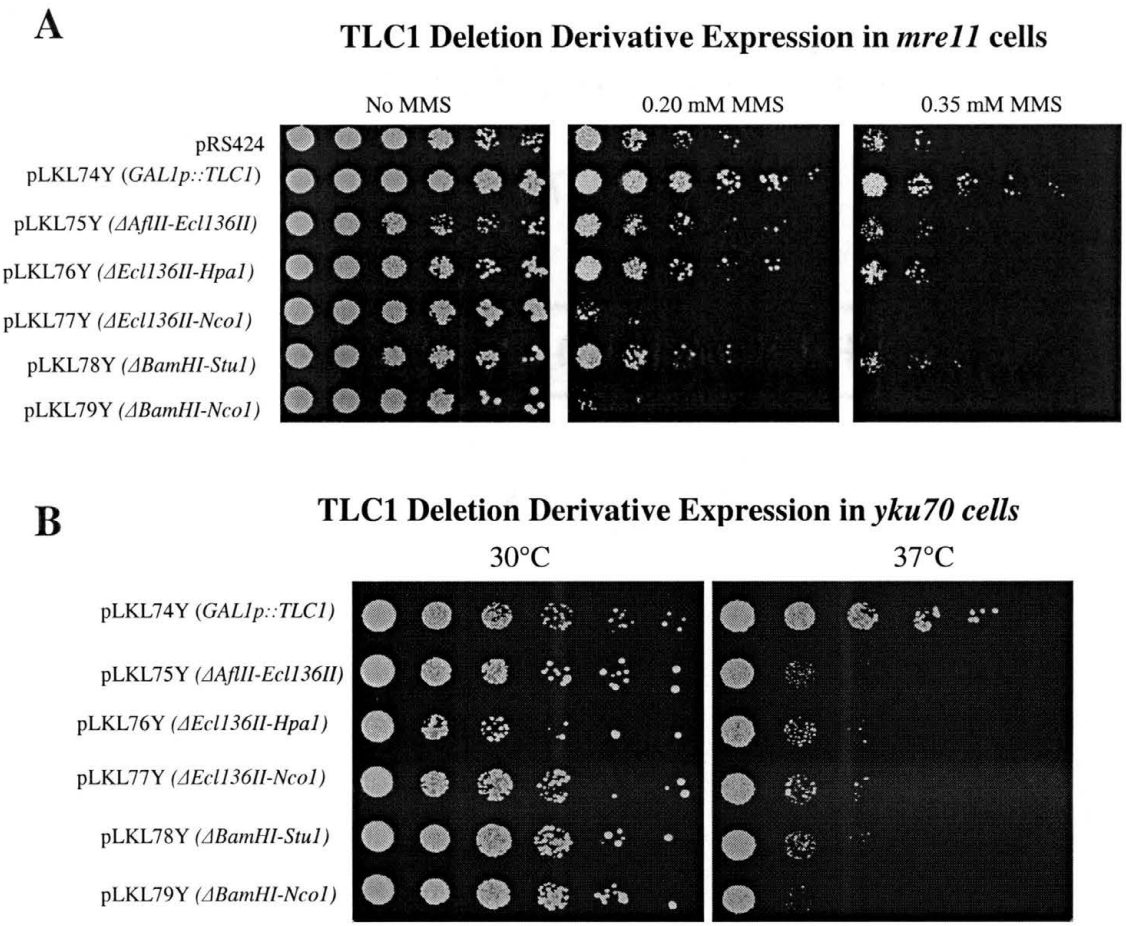


Figure 14. A.) Overexpression of *TLC1* deletion derivatives did not suppress MMS induced killing. B.) Survival did not increase at 37° C upon overexpression of *TLC1* deletions. YLKL593 (*yku70*) was 5-fold dilution pronged onto 2%Glu+2%Gal media and grown at 37° C.

As seen in Figure 14A, *TLC1* deletion derivatives failed to rescue RMX mutants at concentrations of MMS as low as 0.20 mM MMS. Furthermore, two of the derivatives, pLKL77Y and pLKL79Y, actually reduced survival of the *mre11* mutants. Each of these two derivatives contains the largest deletion, from the 5' end and 3' end, respectively. The result involving pLKL79Y (stem/template$^-$) is also consistent with previous studies which have suggested a high level of importance to each of these

regions. The stem loop may be important for recruitment of other telomerase associated components (18) and the template region is needed for DNA elongation at the telomeres.

A TLC1 derivative specifically lacking the template region does not rescue MMS-induced killing

The short region within *TLC1* RNA that provides a substrate for the remainder of the complex to perform the elongation of chromosome ends is known as the template region and consists of multiple CACA repeats. Our laboratory has obtained (as a gift from Dan Gottschling) a plasmid containing *GAL1p::TLC1-ΔCA* (pTCGΔCA) which is a *TLC1* derivative lacking the 17 nt template region. pTCGΔCA can be used to assay whether templated DNA replication by telomerase is necessary for the increased DSB repair in RMX mutants. To pursue this question, pTCGΔCA was transformed into YLKL783 (*mre11-T334*). The cells were assayed by 5-fold dilution pronging onto media (2% glucose + 2% galactose) containing increasing concentrations of MMS (Figure 15).

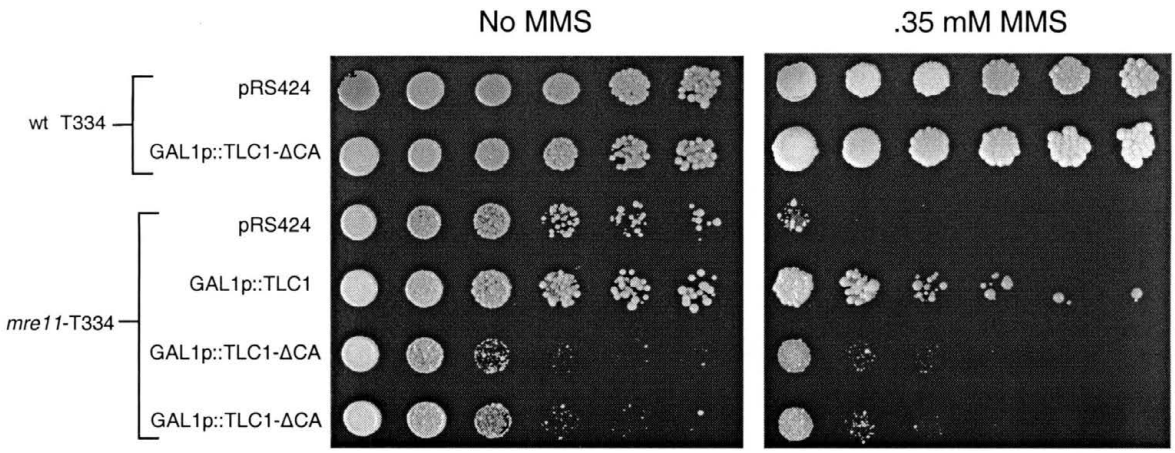


Figure 15. Overexpression of *TLC1*-ΔCA does not suppress MMS-induced killing compared to *TLC1*.

As shown in the figure, the template-deficient mutant (TLC1-ΔCA) did not rescue MMS-induced killing, and was also toxic to *mre11* cells grown in normal media without MMS (two independent isolates are shown). *TLC1-ΔCA* RNA is likely to be associated with the proteins of the telomerase complex. Such complexes would be expected to still be able to bind to the ends of chromosomes because Est1 and Cdc13 are DNA end-binding proteins. However, these complexes would be unable to synthesize new DNA. The toxicity of *TLC1-ΔCA* RNA expression (Figure 15) is likely due to the presence of such inactive enzyme complexes though the exact mechanism is unclear. Most importantly, these experiments established that the 17 nt template region is essential for enhancement of DNA repair in RMX mutants.

Expression of the stem-loop region of TLC1 rescues MMS induced killing

Along with the template region, *TLC1* RNA contains a 48 nt stem-loop region located in the 5' end (Figure 16A). A recent study has implicated the stem-loop in *TLC1* RNA overexpression-mediated alleviation of telomeric silencing and interaction with DNA end-binding Ku proteins (18). Our laboratory wanted to determine if overexpression of the stem-loop could rescue RMX mutants. To address this question, a plasmid containing *GAL1p::3xSTEM* (pTLC1-3xSTEM), which contains three consecutive copies of the 48 nt stem-loop region was utilized (Figure 16B). Six separate isolates of pTLC1-3xSTEM along with pRS314 and pTRP61 (*TLC1*) were transformed into YLKL503 cells (*Δmre11*-VL6α). The resulting transformants were plated by dilution pronging onto galactose media containing 0.35 mM MMS (data not shown).

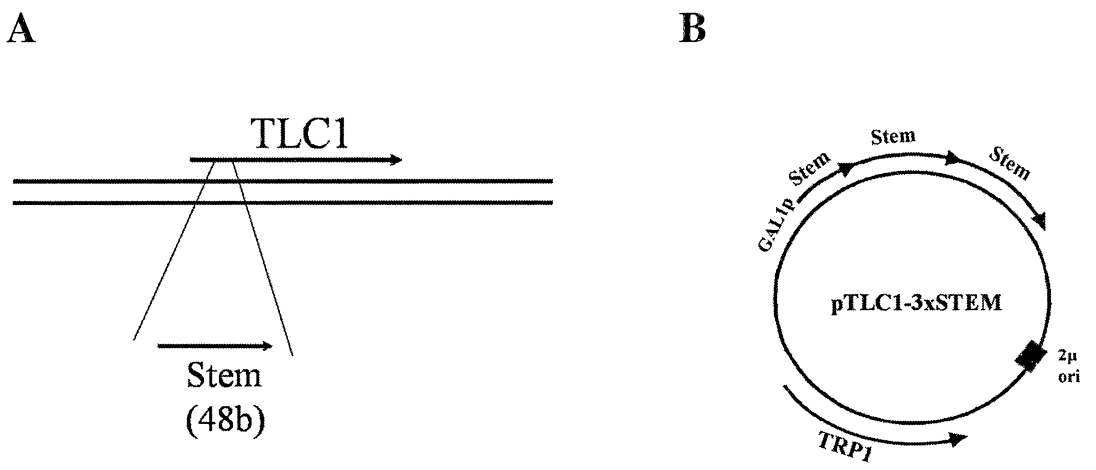


Figure 16. A.) Schematic showing 48nt stem-loop region in the 5' end of *TLC1* RNA. B.) Plasmid diagram of pTLC1-3xSTEM showing three stem-loop repeats under the control of a *GAL1* promoter.

Analysis of the plates revealed ambiguous results in that three of the stem-loop isolates rescued the *mre11* mutants while the remaining three failed to rescue. To follow up this result, we tested a second set of 16 different stem-loop plasmid transformants (Figure 17).

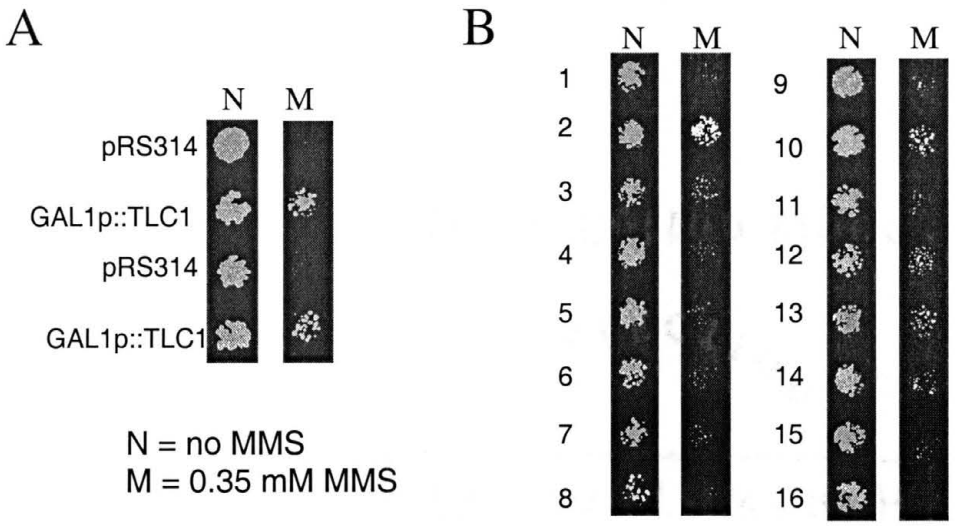


Figure 17. A.) Controls indicating *TLC1* RNA rescue of *mre11* mutants. B.) Four stem-loop isolates (2, 10, 12 and 13) were able to rescue (inefficiently) *mre11* mutants when exposed to 0.35 mM MMS.

Again, this experiment was carried out by dilution pronging onto MMS containing media, and as seen in Figure 17, analysis of the plates showed approximately 25% (four isolates) rescued *mre11* mutants. This result prompted questions about plasmid stability *in vivo*. Since pTLC1-3xSTEM consists of three homologous repeats, the repeats can recombine within themselves leading to deletion of one or more of the repeats. A deletion of one repeat would decrease the total size of the plasmid. This

allowed us to investigate the possibility of plasmid instability as the cause of our ambiguous results. To test for deletions within the plasmid, T3 and T7 primers were used to analyze wildtype *TLC1* (pCDNA50.3), three separate stock stem-loop preparations (pTLC1-3xSTEM A, 1 and 2), as well as plasmid DNA isolated from two MMS resistant and two MMS sensitive plasmid transformants (Figure 18).

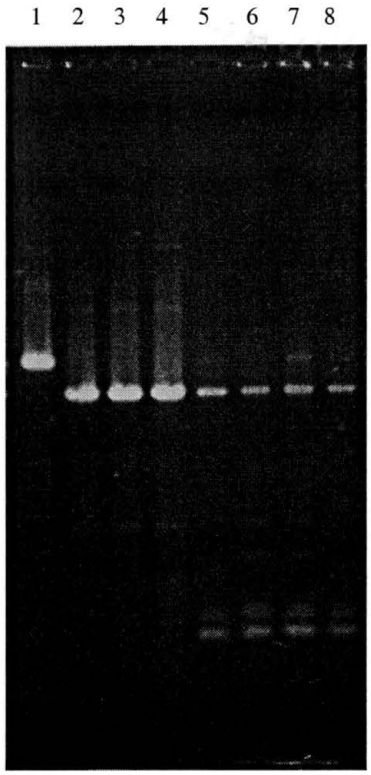


Figure 18. PCR analysis of various pTLC1-3xSTEM isolates. Lane 1: wildtype *TLC1*; Lanes 2, 3 and 4: stock pTLC1-3xSTEM a, 1 and 2 respectively; Lanes 5 and 6: pTLC1-3xSTEM isolates from MMS resistant cells (isolates 2 and 10 respectively); Lanes 7 and 8: stem-loop isolates from MMS sensitive cells (isolates 1 and 9), respectively.

As seen in Figure 18, PCR analysis of stem-loop isolates from MMS sensitive cells (lanes 7 and 8) did not show a reduction in size compared to MMS resistant cells (lanes 5 and 6) or stock preparations of purified pTLC1-3xSTEM plasmid (lanes 2, 3 and 4). This result is a strong indication that the plasmids from sensitive cells were not altered resulting in shorter length. Presumably, the full length of the three repeats was expressed.

The PCR result ruled out the possibility of deletions in the stem-loop isolates. The next question addressed was whether the results being observed with the stem-loop isolates were a strain-specific phenomenon. In order to test this question, 12 separate pTLC1-3xSTEM isolates were transformed into *mre11*-T334 cells (YLKL783). This strain possesses the same deletion as the previously tested YLKL503 (*mre11*-VL6α) except in a different strain background, T334. Transformants were plated by dilution pronging onto 2% glucose + 2% galactose media containing 0.35 mM MMS (Figure 19).

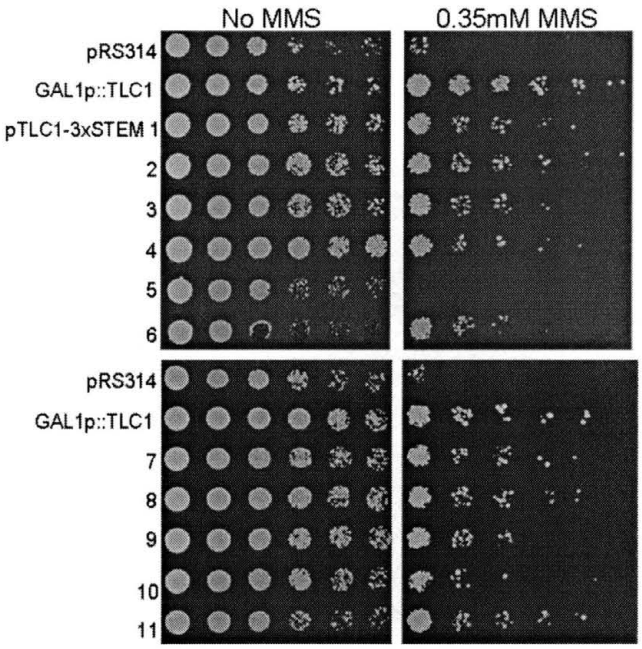


Figure 19. Overexpression of pTLC1-3xSTEM rescues *mre11*-T334 cells. A total of 12 independent pTLC1-3xSTEM plasmid fragments were assayed.

Overexpression of pTLC1-3xSTEM was able to suppress MMS-induced killing in 11 of 12 *mre11*-T334 transformants (Figure 19). One explanation for this result compared to experiments performed with *mre11*-VL6α may be that the stem-loop RNA is present at a higher concentration in *mre11*-T334 cells. Since it has been suggested that the stem-loop may bind telomere proteins (18, 19, 20), higher concentrations of the RNA may titrate these proteins away from broken DNA ends exposing the ends for repair complexes. A second explanation might be that the RNA containing repeats have decreased half-lives within *mre11*-VL6α cells. RNA molecules are present for only a short time *in vivo*. Early decomposition would decrease the time the RNA had to interact and titrate away DNA end-binding proteins.

TLC1 RNA overexpression causes toxicity in nuclease-deficient mre11-D16A cells

Mre11 is the nuclease component of the RMX complex. This protein, as well as Rad50 and Xrs2, binds to broken DNA ends and performs an important task by forming 3' overhangs in the initial step of homologous recombination. To investigate the role of the nuclease activity of RMX, a nuclease-deficient form of Mre11 was created to express Mre11-D16A. In a recent study conducted in our laboratory, *mre11-D16A* cells exhibited similar recombination deficiency to *mre11* null mutants and were suggested to be required for RMX-mediated repair of DSBs (Lewis *et al.* in press, April 2004). Furthermore, *mre11-D16A* mutants retained the ability to form a complete RMX complex and bind to broken DNA ends (32). To determine whether overexpression of *TLC1* RNA would suppress MMS-induced killing in *mre11-D16A* mutants, strain YLKL641 (*mre11-D16A*) was transformed with cloning vector pRS314 (*CEN/ARS TRP1*) and pTRP61 (*2μ TRP1 GAL1p::TLC1*). Transformants were assayed by dilution pronging onto galactose media containing 0.35 mM MMS (Figure 20).

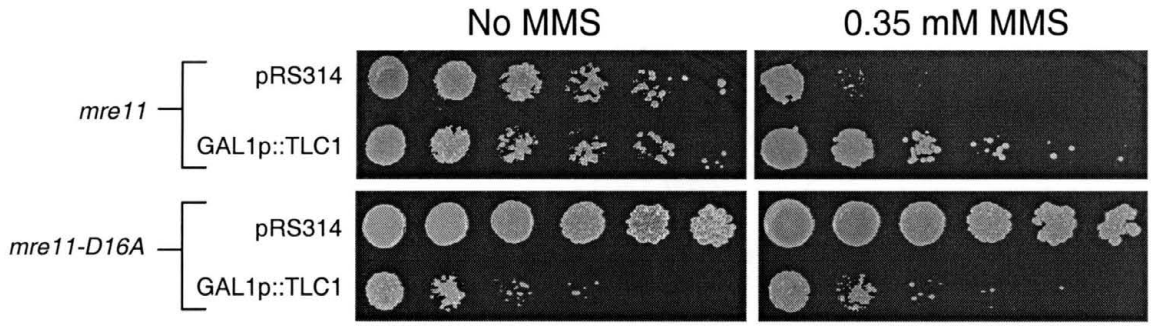


Figure 20. Overexpression of *TLC1* RNA is toxic in *mre11-D16A* mutants on galactose media without MMS. Growth was not inhibited on glucose media.

As seen in Figure 20, *TLC1* overexpression causes toxicity in *mre11-D16A* cells, decreasing survival even without exposure to MMS. Growth was not inhibited on glucose media, which does not activate the *GAL1p*. One explanation for this toxicity is that the mutant Rad50/Mre11-D16A/Xrs2 complex binds to the ends of chromosomes and is unable to process the ends, inhibiting the access of telomerase and possibly DNA end-binding Ku proteins. Normally, telomerase and Ku proteins may bind before or displace the nuclease deficient RM*X complex, but these proteins are titrated away in the presence of elevated levels of *TLC1* RNA. A recent study suggests that YKu70 protein binds to the stem region of *TLC1* RNA (18). An interesting question is whether overexpression of *YKU70* from a plasmid can alleviate the titration effect of *TLC1* RNA. It is possible that high levels of YKu70 protein may create elevated competition for chromosome ends allowing access to telomerase proteins.

Summary and Conclusions

Results obtained with *Eco*RI expression *in vivo* indicate that *TLC1* RNA is specifically suppressing the negative effects from the presence of DSBs. Also, the NHEJ repair pathway is not necessary but homologous recombination is essential for *TLC1* RNA rescue of RMX mutant cells. The latter conclusion was proposed because overexpression of *TLC1* RNA did not suppress MMS-induced killing in *mre11 rad52* (Rec^-) cells indicating the requirement for homologous recombination.

TLC1 RNA overexpression did not suppress MMS-induced killing in *mre11 exo1* double mutants. This result reinforces the findings of a previous study done in our laboratory indicating that overexpression of *EXO1* enhanced repair in RMX mutants (21). In the absence of RMX, *EXO1*, which codes for a 5'-3' exonuclease, may substitute poorly for the missing nuclease activity because competing end-binding proteins prevent access or process the ends incorrectly.

Results obtained with several *TLC1* RNA deletion derivatives indicated that even a small deletion of the RNA inhibits its ability to suppress MMS-induced killing. Moreover, the stem-loop and template regions were also necessary for this suppression, possibly to bind interfering DNA end-binding proteins and/or to maintain proper secondary structure. Along this same line, a derivative of *TLC1* RNA missing the 17 nt template region caused toxicity in *mre11* cells likely due to the inability to synthesize new DNA. *TLC1* RNA derivatives containing only the stem region displayed a strain-specific suppression of MMS-induced killing. Finally, *TLC1* RNA caused toxicity in nuclease-deficient *mre11-D16A* cells on media without MMS making it difficult to

discern its ability to suppress MMS-induced killing. The toxicity is likely due to the ability of Mre11-D16A protein to form the complete RMX complex and bind DNA ends unproductively.

What might *TLC1* RNA be doing to enhance repair of DSBs via homologous recombination? Previous studies have indicated that *TLC1* RNA is bound by several proteins. For example, telomerase proteins Est1 and Est2 have been shown to bind to a large central region within the RNA (Figure 21) (19). In addition, the YKu70/YKu80 protein complex is suggested to bind to the 48 nt stem region at the 5' end of the RNA (18). Furthermore, a previous study indicated that *TLC1* RNA is associated with proteins of a snRNP (small nuclear ribonucleoprotein particle) (20).

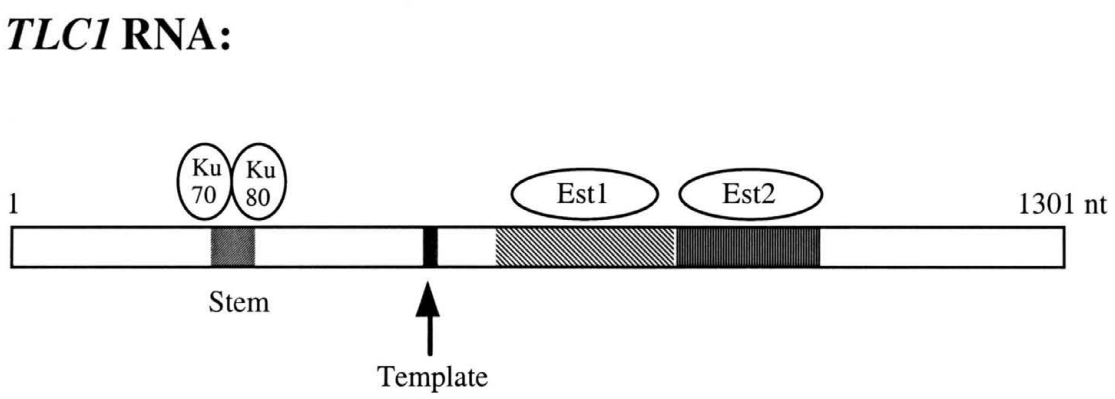


Figure 21. Diagram indicating protein binding sites on *TLC1* RNA.

We found that *TLC1* RNA derivatives containing three copies of the stem region, which as stated above is a binding site for the Ku protein complex, suppressed MMS-induced killing in RMX mutants. This result suggests a possible model in which the

suppression of MMS-induced killing may be caused by the many small *TLC1* RNAs being bound *in vivo* by proteins such as YKu70/YKu80, or conceivably other telomerase proteins, and in some way increasing repair by homologous recombination.

Figure 22 represents our proposed model for *TLC1* RNA-mediated repair in RMX mutants. As seen in Figure 22A, in wildtype cells broken ends are normally processed by RMX with some backup by Exo1. In contrast, broken DNA ends are primarily processed, though less effectively, by Exo1 in RMX-deficient cells. We propose that there are one or more competitive DNA end-binding proteins that may interfere with Exo1 and possibly with the Rad52 recombination machinery (Figure 22B). YKu70/YKu80 is a likely candidate for such an end-binding protein. In the same RMX-deficient cells when *TLC1* RNA is overexpressed, the many RNA molecules may sequester the competitive end-binding proteins, restoring access to Exo1 or the Rad52 recombination apparatus and ultimately leading to enhanced recombinational repair (Figure 22C).

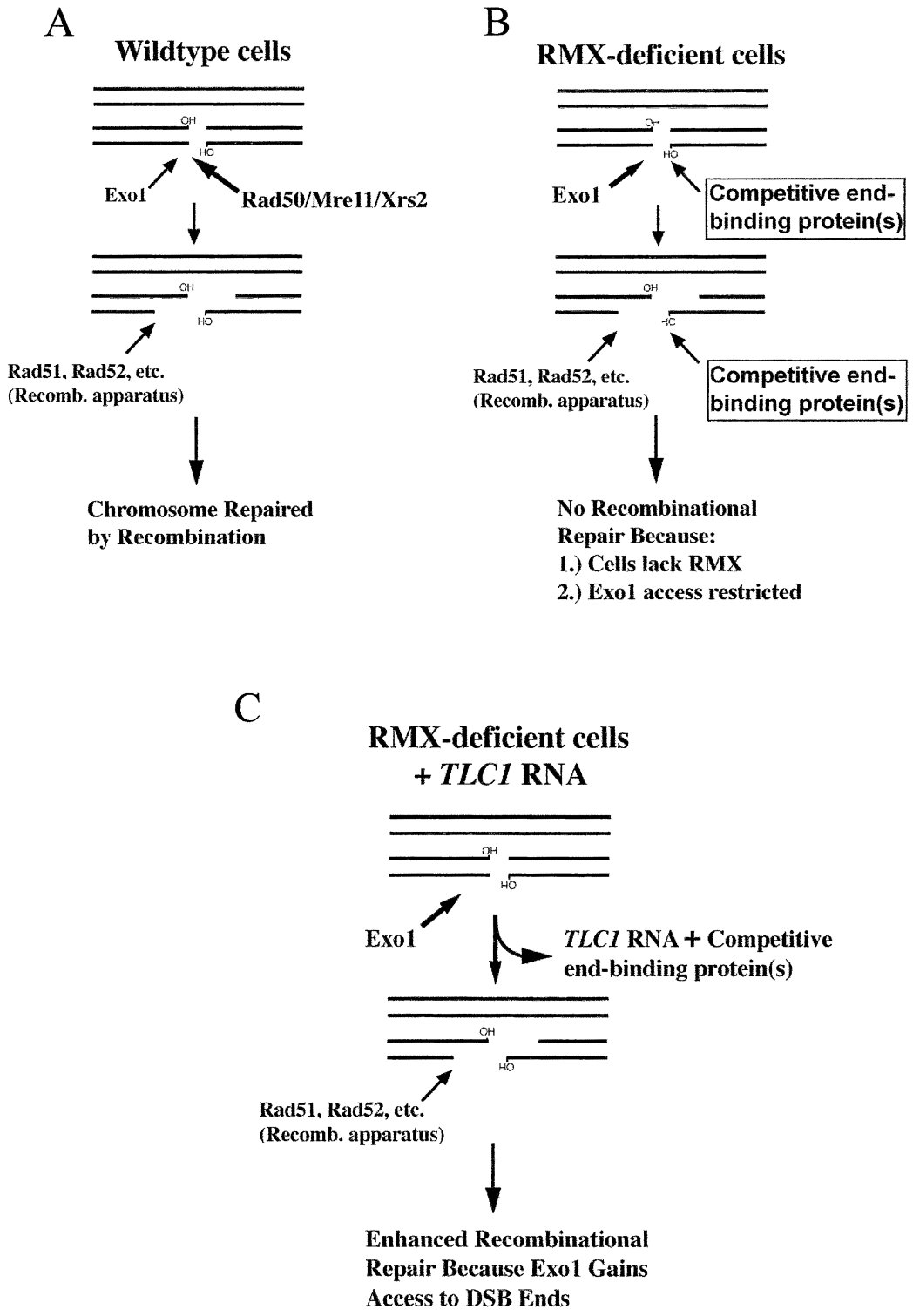


Figure 22. Models for homologous recombination in (A) wildtype cells, (B) RMX-deficient cells and (C) RMX-deficient cells overexpressing *TLC1* RNA.

In conclusion, larger implications of this study include a greater understanding of telomerase proteins and their involvement in DNA repair, insight into RMX function in repair of DSBs and possibly new interactions between DNA-end binding proteins and telomerase subunits. Ultimately, results obtained in this study could advance our understanding and treatment of cancer and aging.

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