REPAIR OF DNA DOUBLE-STRAND BREAKS IN CHROMOSOMES: IMPACT OF TELOMERASE

THESIS

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INTRODUCTION

In eukaryotes, the library of genetic information necessary to sustain life is stored in long, linear chromosomal DNA molecules inside the nucleus of a cell. The nucleotide monomers, along with the sugar-phosphate backbone of DNA, contain functional groups that are susceptible to chemical alteration via several reactions. Examples of these functional groups include hydrogen bonds, carbon-carbon and carbon-nitrogen double bonds, carbonyl groups and phosphodiester bonds. Chromosomal DNA is continuously subjected to damage by various factors, either endogenous (e.g. oxidation, deamination, endonuclease activity, genetic disorders, etc.) or exogenous (e.g. radiation or chemical clastogens such as bleomycin and methyl methanesulfonate). Of particular concern is a common type of lesion in the DNA double helix referred to as a double-strand break (DSB) involving cleavage of nearby phosphodiester linkages in both strands. Accurate repair of DSBs results in a healthy, viable cell. There are several undesired consequences of double strand breaks which include no repair, inaccurate repair, translocations or aberrant recombination events, "capping" by de novo telomere formation, and indirect lethality or apoptosis. The latter events may in turn lead to cell death, altered cell metabolism, or in some cases, the development of cancer.

Nature has provided several pathways and enzymes that specialize in maintaining the integrity of chromosomes. There is little that is more vital to a cell's survival than an intact genome. Although there are multiple DNA repair pathways, eukaryotic organisms repair double-strand breaks by two major conserved pathways called homologous recombination and nonhomologous end-joining (NHEJ).

The pathways of homologous recombination and NHEJ have been worked out in the greatest detail in the model eukaryote *Saccharomyces cerevisiae. S. cerevisiae* cells (budding yeast) have characteristics that make them an asset in the field of molecular genetics. One of the first organisms whose genome has been completely sequenced, sophisticated molecular biology techniques have been developed that have made it the organism of choice for many genetic studies. For example, yeast strains exist as either haploids or diploids, contain over 1000 genetic markers, can be efficiently transformed with DNA from external sources, and have short generation times, allowing for rapid analysis (1). Also, yeast cells contain many genes that are highly conserved among higher eukaryotes, making this an ideal model for studying biological processes common to all eukaryotes, including human cells (2).

In *S. cerevisiae*, the NHEJ pathway consists of protein complexes Yku70/Yku80, Rad50/Mre11/Xrs2, Sir2/Sir3/Sir4, and Dnl4/Lif1/Nej1 (Figure 1). The homologous recombination pathway consists of protein complexes Rad50/Mre11/Xrs2 and Rad51/Rad52/Rad54/Rad55/Rad57, as well as several additional less well characterized proteins. The Rad50/Mre11/Xrs2 (RMX) complex is unique because it is required for both of these pathways.

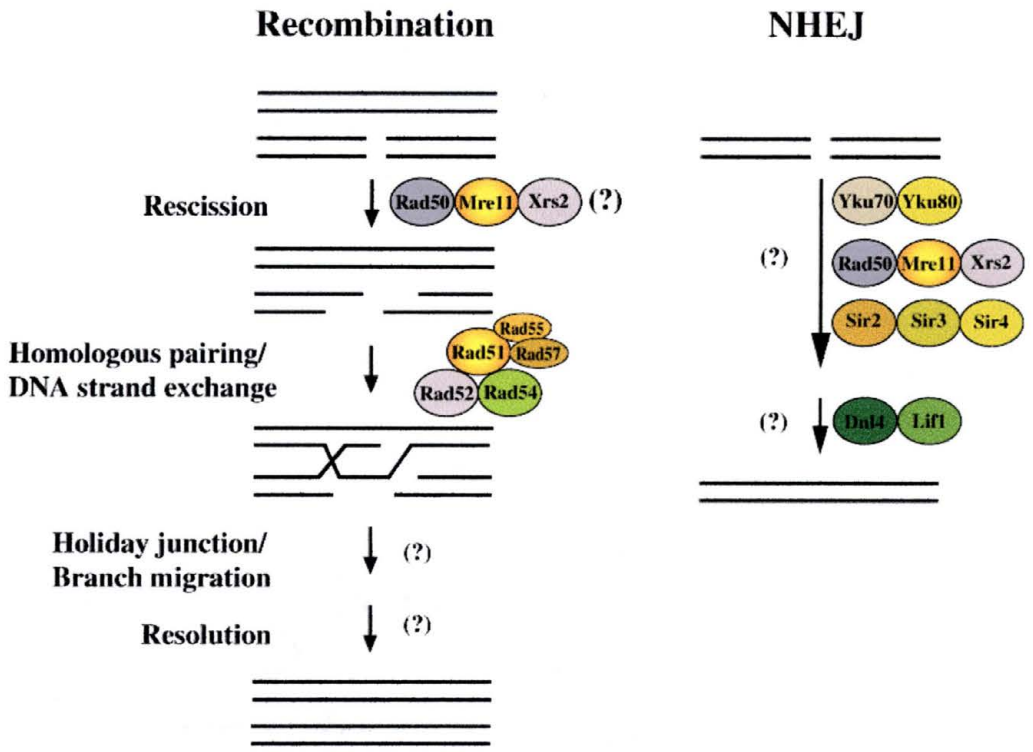


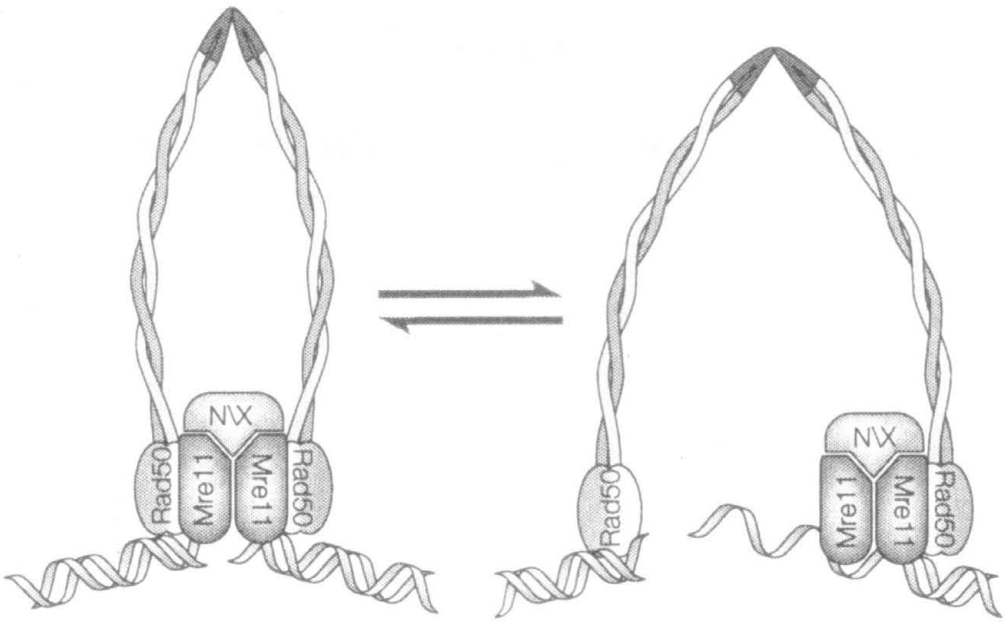
Figure 1. Schematic of yeast recombination and non-homologous end-joining pathways.

The Rad50 protein component is a large ATPase enzyme that binds Zn^{2+} and Mg^{2+} and may function in loading the RMX complex onto DNA (3). Rad50 is related to the SMC proteins which contain Walker A and B motifs separated by a long coiled-coil region and are required for sister chromatid cohesion (4). Studies by electron microscopy and x-ray crystallography suggest that Rad50 forms a folded structure that brings together the Walker A and B motifs, forming two catalytic sites (5, 6).

Mre11 is a manganese-dependant 3'→5' dsDNA exonuclease and ssDNA endonuclease that contains five highly conserved amino-terminal phosphoesterase motifs,

as well as two carboxy-terminal DNA binding domains (3, 7, 8, 9). Mre11 also plays an important role in the S-phase checkpoint response to DNA damage (4, 8). The function of Xrs2 remains unknown, though recent observations that Xrs2 protein physically associates with Lif1 provides a link between RMX and repair by NHEJ (9).

Recent crystal structure studies of *P. furiosus* Rad50 and Mre11 have resulted in a proposed "headphone" model for binding of the RMX complex to broken DNA ends (Figure 2). This work and other genetic studies show that the Mre11/Rad50 complex could function specifically in bridging sister chromatids on broken DNA ends during double-strand break repair. RMX may bind to broken DNA ends and hold them in proximity to each other, allowing Mre11 to excise one strand to leave a 3'overhang, which will serve as a substrate for subsequent repair protein complexes to bind. While the specific mechanism by which the RMX complex mediates DSB repair, activates checkpoints, inhibits chromosome rearrangements and functions at stabilizing chromosome ends remains unclear, several nuclease mutants of Mre11 have been created and are under investigation in an attempt to elucidate these unknown areas.



DSB ends are held in proximity by RMX

Resection of one strand to produce a 3' overhang

Figure 2. "Headphone" model for the binding of the RMX complex to double strand breaks.

In yeast, inactivation of any of the three genes *RAD50, MRE11* or *XRS2* leads to several defects in DNA metabolism. These mutants are defective in both recombination and NHEJ (10). Other phenotypes include extreme sensitivity to chemical and physical clastogens that cause DNA strand breaks (i.e. x-rays, bleomycin, MMS, *Eco*RI endonuclease) increased frequencies of gross chromosome rearrangements, shortened chromosome ends (telomeres) and impairment of DNA damage responsive cell cycle checkpoints (11). In humans, the equivalent complex is named hRAD50/hMRE11/hNBS1. Mutations in hNBS1 and hMRE11 lead to the human genetic disorders Nijmegen Breakage

Syndrome and Ataxia Telangiectasia-like Disorder (ATLD), respectively. The phenotypes of both disorders include extreme sensitivity to radiation and an increased incidence of cancer (4, 8).

As described above, RMX mutants exhibit telomere instability (i.e. the ends of chromosomes in these cells are shorter than in wildtype cells). Interestingly, another connection between RMX and telomeres was recently observed in this laboratory (12). In a genetic screen performed to identify genes that could rescue the extreme MMS sensitivity of *rad50* cells when overexpressed, two genes, *EXO1* and *TLC1*, were identified. *EXO1* encodes a 5' to 3' DNA exonuclease previously implicated in mismatch repair, DNA replication, and recombination.

The second gene, *TLC1*, encodes the RNA template component of the yeast telomerase complex. Telomerase is a conserved RNA-dependent DNA polymerase that extends the ends of chromosomes during S-phase. During each cell cycle, telomerase adds a short, repeated sequence to the chromosome ends (See Figure 3). These noncoding repeat sequences form the telomeric regions at the ends of the chromosomes. A typical yeast telomere contains ~300 bp of $TG_{(1\text{-}3)}$ repeat sequences. The RNA component of the complex (*TLC1*) contains 17 base template region (5'-CACCACACCCACACACA-3') that contains the information needed to synthesize the new strand. The yeast telomerase complex consists of at least four proteins including Est1, Est2, Est3 and Cdc13, and also includes *TLC1* RNA. Mutations in the *EST1, EST2, EST3* or *CDC13* genes lead to progressive telomere shortening and eventual cell senescence. Cdc13 and Est1 proteins can bind to single-stranded G-rich telomeric sequences such as those found at the end of chromosomes during S phase (13, 14). Other

proteins that appear to be associated with the complex include Ten1 and Stn1. The Est2 subunit has been shown to have the polymerase (catalytic) activity of the complex and this enzyme contains five highly conserved motifs common to many reverse transcriptase enzymes (16).

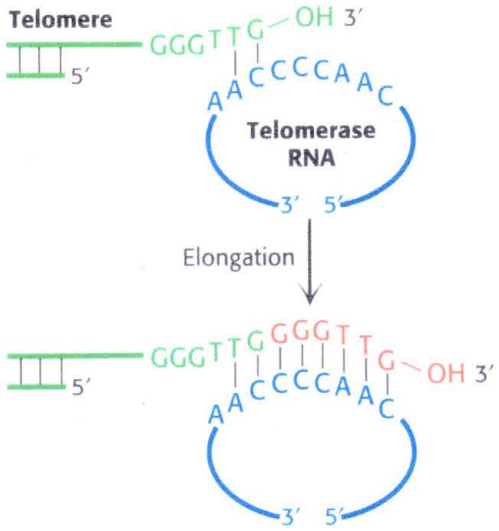


Figure 3. Telomere elongation. General mechanism for templating and elongation of the 3' end of a chromosome by telomerase RNA and telomerase.

Human or yeast cells that lack telomerase experience shortening of telomeres over time, increased chromosome instability and cell death. In humans, production of the telomerase enzyme halts in most cell types during embryonic development. Only highly proliferative cells, such as germ cells, continue expressing low levels of the enzyme (16, 17). In mammalian cell culture, cells in which telomerase is being expressed show "immortal" growth. In contrast, normal cells that are not actively expressing telomerase typically go through an average of 50-60 replications before experiencing cellular

senescence. These findings support the role telomerase is believed to play in cancer, which represents the unchecked division of cells that have acquired mutations that abolish normal growth control. In order to support unlimited divisions, these cells need a way to avoid the normal shortening of chromosome ends. It has been observed that in over 90% of cancers, telomerase has been reactivated (16). An exception to this is stage IV neuroblastoma in children and infants, which in 85% of cases shows regression with no intervention. Recent studies of biopsies from children who have recovered showed that all of the tumors exhibited very low telomerase activity (18). This suggests that regression of stage IV neuroblastoma is caused by the absence of telomerase in combination with telomere shortening. The unchecked division of these cells, which are not actively expressing telomerase, results in telomeres becoming shorter and shorter and increasingly unstable, and ultimately leads to cellular senescence (19). Therefore, the rapid division of these cancerous tumors has a finite limit. Its direct implication in cancer has made telomerase a popular topic of research in the past eight years. Current research focuses on finding a way to inactivate telomerase in cancer cells mimicking the outcomes seen in stage IV neuroblastoma, and along with use of current anti-cancer therapies, to reinstate a finite cellular lifespan (20). Another area that cellular telomerase levels may affect is aging. A recent statistical study of an elderly group showed that people with short telomeres died nearly twice as fast as people with longer telomeres (21).

Several experiments by Lewis *et al.* (12) and Ogawa and Tsubouchi (22) demonstrated that Exo1 overexpression specifically increases recombinational repair of DSBs in *rad50, mre11* and *xrs2* mutants. No effects on the NHEJ repair pathway were observed in these studies. These studies suggest that the nuclease activity of Exo1 is able

to substitute for the RMX nuclease complex in processing of broken DNA ends to generate a 3' tail that can serve as a substrate for the homologous recombination apparatus (Rad51, Rad52, Rad54, Rad55, Rad57, etc.).

In contrast to Exo1, the mechanism by which *TLC1* RNA overexpression increases resistance to MMS and X-rays in RMX mutants remains unclear. In an effort to explore this observation, three possible mechanisms have been proposed by our lab. First, it may be that overexpressing components of telomerase results in an increase in recombinational repair. Related, is the idea that increased resistance results from an increase in end-joining repair (NHEJ). Finally, it may also be possible that *de novo* telomere addition may be occurring at the site of double-strand breaks, therefore stabilizing chromosomes.

The complete mechanism by which the RMX nuclease complex functions in the resolution of DSBs in recombination and NHEJ remains unclear. The goal of the work described herein was to learn more about the telomerase complex and its relationship to DSB repair, and is also an effort to further elucidate the role of the Rad50:Mre11:Xrs2 complex in DSB repair. The ability of *TLC1* RNA to increase resistance to DNA damaging agents has led to many interesting questions. Specifically, what role does telomerase play in DSB repair, and what other protein components of the telomerase complex make cells more resistant to DNA damage? Information from these and additional experiments have been used to evaluate the mechanism by which these components affect DNA repair. In addition, another part of this project has involved an assessment of the role of the nuclease activity of Mre11 in repair of DSBs.

CHAPTER 2

MATERIALS AND METHODS

I. MATERIALS

General Reagents

Ammonium sulfate (granular), sodium chloride and sodium dodecyl sulfate were purchased from Mallinckrodt AR (Paris, Kentucky). Agarose and ethidium bromide were purchased from Shelton Scientific, Incorporated (Shelton, CT). Hydroxyurea (HU) was purchased from US Biological (Swampscott, MA). Methyl methanesulfonate (MMS) was obtained from Fluka. Lithium acetate dehydrate, calcium chloride, 99% glycerol, polyethylene glycol, 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). Sodium citrate dehydrate and sodium hydroxide were purchased from EM Science (Gibbstown, NJ). Maleic acid and formamide were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Reagents used for immunodetection of Southern blots were all obtained from Roche Diagnostics Co. (Indianapolis, IN).

Bacteriological and yeast media

All amino acids, plate agar, D-(+)-glucose, ampicillin, and galactose were purchased from Sigma Chemical Co. (St. Louis, MO). Difco bacto peptone, bacto yeast

extract, bacto tryptone and bacto yeast nitrogen base dropout 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, nonselective 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). Hydroxyurea (HU) plates were prepared by using synthetic media plus aliquots of a stock solution of 1M HU solution mixed to obtain various concentrations of HU. Methyl methanesulfonate (MMS) plates were made using synthetic media or YPD plus Fluka MMS mixed to obtain various concentrations.

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
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	LAB STRAIN
T334	*MATα ura3-52 leu2-3,112 Δtrp1::hisG reg1-501 gal pep4-3 prb1-1122*	(12)
YLKL350	T334, *Δhis3..[GAL1::EcoRI TRP1]*	(12)
YLKL407	YLKL350, *Δmre11::G418^r*	(12)
VL6α	VL6α, *MATa*	LARIONOV *et al.* (1994)
VL6-48	VL6α, *Δleu2::G418^r*	LAB STR AIN
YLKL276	VL6α, *Δrad52..hisG*	(12)
YLKL483	VL6-48, *Δsir4::LEU2*	LAB STR AIN
YLKL503	VL6α, *Δmre11::G418*	(12)
YLKL512	YLKL483, *Δrad51::hisG*	LAB STR AIN
YLKL544	VL6-48, *Δrad50::hisG-URA3-hisG*	LAB STR AIN
YLKL532	VL6α, *Δrad51::hisG*	(12)
YLKL593	VL6-48, *Δyku70:.HIS3*	LAB STR AIN
YLKL596	YLKL593, *Δrad51::hisG-URA3-hisG*	LAB STR AIN
YLKL601	YLKL544, *Δrad51::hisG-URA3-hisG*	LAB STR AIN
YLKL603	YLKL596, *Δrad51::hisG*	LAB STR AIN
YLKL613	YLKL544, *Δmre11.:HygB*	LAB STR AIN
YLKL650	BY4742, *Δmre11:.G418^r*	LAB STR AIN
YLKL608	YLKL601, *Δrad51·.hisG*	LAB STR AIN
YLKL612	VL6α, *Δsir4:.LEU2, but Δmre11::HygB*	LAB STR AIN

Table 2. Plasmids

Plasmid	Description	Referencs/Source
pCDNA50.3	*CEN/ARS URA3 GALp::TLC1*	(12)
pHT234	*Δmre11::URA3*	K. LOBACHEV
pMre11-D16A	*CEN/ARS TRP1 mre11-D16A*	This lab
pRDK480	*2 μ LEU2 EXO1*	TISHKOFF *et al.* (1997)
pRS314	*CEN/ARS TRP1*	SIKORSKI AND HIETER (1989)
pRS315URA3	*CEN/ARS URA3 LEU2*	This lab
pSM258	*CEN/ARS TRP1 MRE11*	MOREAU *et al.* (1999)
pSM304	*CEN/ARS TRP1 mre11-H125N*	MOREAU *et al.* (1999)
pSM312	*CEN/ARS TRP1 mre11-D56N*	MOREAU *et al.* (1999)
pVL459	*2 μ URA3 CDC13*	(23)
pVL715	*2 μ URA3 ADH1p::EST2*	(24)
pVL735	*2 μ URA3 ADH1p::est2-D530A*	(24)
pVL743	*2 μ URA3 ADH1p::est2-D670A*	(24)
pVL744	*2 μ URA3 ADH1p::est2-D671A*	(24)
pVL999	*2 μ LEU2 ADH1p::EST2*	(24)
pVL1035	*2 μ TRP1 GAL1p::STN1*	(23)
pLKL64Y	*2 μ LEU2 ADH1p::TLC1*	This lab
pTRP61	*2 μ TRP1 GAL1p::TLC1*	(25)
YEp-195-TEN1	*2 μ URA3 TEN1*	(14)
YEp-195-STN1	*2 μ URA3 STN1*	(14)
YTCA-1	$TG_{(1-3)}$ repeat plasmid	(26)

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 (27).

Yeast transformations

Yeast transformations were performed using either a high efficiency method described by Gietz *et al.* (28) or a rapid DMSO-based transformation method by Soni *et al.* (29).

Dilution pronging survival assay

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 onto plates containing either varying concentrations of DNA damaging agents (i.e. MMS, HU) or media containing 0.2% 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.

MRE11 gene disruption

mre11::hisG-URA3-hisG disruptions were generated using plasmid pHT234, digested with restriction enzymes *Pvu*II and *Swa*I. The cut plasmid was transformed into YLKL475. This transformation was done for the purpose of deleting the *MRE11* gene in YLKL475. The transformants were grown on Glu-Ura at 30 °C for two days. Individual colonies were patch purified and then replica-plated onto YPG, YPD and YPD + 1mM MMS. Transformants that were determined to have the *MRE11* gene deleted were identified by their MMS sensitivity. Images of the plates were captured using a digital camera.

Southern Blots

PCR amplification of nonradioactive DIG probe. Probes for Southern analysis were synthesized by PCR. Two reactions were performed using a prep of plasmid YTCA-1 that was diluted 1/100 for one reaction and 1/1,000 for the other. One microliter of miniprep template DNA, 5 µl M13 forward primer (5'-AGCGCGCAATTAACCCTCACTAAAG-3'), 5 µl M13 reverse primer (5'-CAGGAAACAGCTATGACC-3'), 5 µl 10X PCR buffer with $MgCl_2$, 5 µl 10X PCR DIG labeling mix (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.3 mM dTTP, 0.7 mM digoxigenin-11-dUTP) and 0.75 µl Taq DNA polymerase enzyme were combined with sterile double distilled water to bring each reaction to a final volume of 50 µl. An unlabeled DNA control was run and the reaction contained all of the same additions as mentioned above only no DIG labeling mix was added. A kit control PCR was also run and varied from the initial reactions described above only by the addition of a control template DNA in place of YTCA-1 template DNA. The reactions were then exposed to

the following program conditions: 94°C, 2 min., and then 32 cycles (95°C for 25 sec., 52°C for 30 sec., 72°C for 30 sec.) followed by extension of all unfinished strands at 72°C for 7 min. The PCR samples were run on a 2.2% agarose gel stained with ethidium bromide and visualized on a Kodak Image Station 440.

DNA isolation and purification. Twenty ml overnight cultures were started in selective liquid media and grown in a 30°C shaker. The DNA was then purified using the MasterPure™ Purification Kit from Epicentre. After purification, DNA concentrations were quantified on a Hoefer DyNA Quant 200 Fluorometer (Amershem Pharmacia Biotech). Three μg of DNA was digested overnight with *Xho*1 at 37°C. The *Xho*1-digested DNA was concentrated by ethanol precipitation and again quantified by fluorometry. One μg of each sample was loaded onto a 1.2% agarose gel and visualized using ethidium bromide.

Denaturation and neutralization. After electrophoresis, the gel was washed with denaturation buffer (8.7% sodium chloride, 2% sodium hydroxide) for a total of 35 minutes at room temperature. Next, the gel was washed in neutralization buffer (8.7% sodium chloride, 6.1% Tris base) for a total or 35 minutes at room temperature. The DNA from the gel was transferred overnight onto an N^+ Hybond membrane using a homemade capillary transfer apparatus. Once overnight transfer was complete, the DNA was crosslinked to the membrane in a UV-Stratalinker 2400 (Stratagene) at 120,000 microjoules for ~20 seconds.

Hybridization. For prehybridization, the membrane was rotated in a glass roller bottle in 15 mL of prehybridization/hybridization solution (50% v/v formamide, 5X SSC, 0.1% sarkosyl, 0.02% SDS, 1X blocking agent from Roche) for one hour at 40°C. 5X

SSC buffer was prepared from 20X SSC stock solution (35.1% NaCl and 17.6% sodium citrate). The 15 mL of prehybridization solution was poured off and replaced with 15 mL of fresh prehyb/hybridization solution. Thirty microliters of digoxigenin (DIG)-labeled probe (2 μL probe/mL hybridization buffer) was added to the tube. The membrane in solution was rolled overnight at 40°C.

Detection by chemiluminescence. The blot was removed from the overnight tube and placed in a solution of 2X SSC/0.1% SDS and shaken at room temperature for five minutes. This step was repeated once. The blot was washed twice for five minutes each in 2X stringency wash solution (0.1M maleic acid, 0.15 M NaCl, 0.3% Tween 20 v/v; pH 7.5). Next, the blot was equilibrated for one minute in wash buffer, which was poured off and replaced with fresh wash buffer. After another minute, the wash buffer was poured off and 100mL of blocking solution was added, allowing the blot to shake in it for 30 minutes. The blocking solution was then poured off and 20 mL of enzyme-linked antibody solution (4 μL antibody solution from Roche in 20 mL 1X blocking solution) was added and the blot was left to shake for 30 minutes. The antibody solution was poured off and the blot was washed twice with 100 mL wash buffer for 15 minutes each. This solution was poured off and the blot was incubated for 2-5 minutes with 20 mL of detection buffer (0.1M Tris-Hcl, 0.1 M NaCl; pH 9.5). The blot was removed from the buffer and placed into a plastic bag. One milliliter of substrate solution containing disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3,3.1.13.7]decan}-4-yl) phenyl phosphate (CSPD), from Roche Diagnostics was added directly onto the blot in the bag. The bag was immediately closed and the substrate solution was spread evenly over the surface of the membrane. The membrane sat at room temperature for five

minutes before all of the liquid was squeezed out of the bag and the bag was sealed. The blot was incubated at 37°C for 10 minutes. For imaging, the blot was placed in an X-ray cassette and with one sheet of 8x10 cm Kodak Biomax MR film. Exposure times for each film varied from 10-120 minutes before development.

CHAPTER 3

RESULTS AND DISCUSSION

The focus of this research project is the Rad50:Mre11:Xrs2 (RMX) complex, which functions in DNA double-strand break repair by the pathways of recombination and nonhomologous end-joining (NHEJ) and is also required for telomere stabilization. The first part of the project involved experiments done to assess the role of the nuclease activity of the RMX complex in repair of DNA damage induced by the chemical clastogens hydroxyurea (HU) and methyl methanesulfonate (MMS). The focus of the second part of the project was to understand how RMX-mediated DSB repair pathways are influenced by components of the telomerase complex.

DNA repair proficiency of Mre11 nuclease-deficient mutants

Previous analyses of putative nuclease-defective Mre11 proteins have produced conflicting results about the requirement for its catalytic activities in mitotic cells. Our laboratory has recently conducted a study of three phosphoesterase mutants of Mre11. These three mutants are the only mutant proteins shown to lack nuclease activity *in vitro* but to still be proficient at other functions of the complex (e.g. RMX complex formation, DNA binding capabilities, etc.) (30, 31). Each of the three mutants, D16A, D56N and H125N, contain single amino acid substitutions within conserved phosphoesterase motifs of Mre11 (Figure 4).

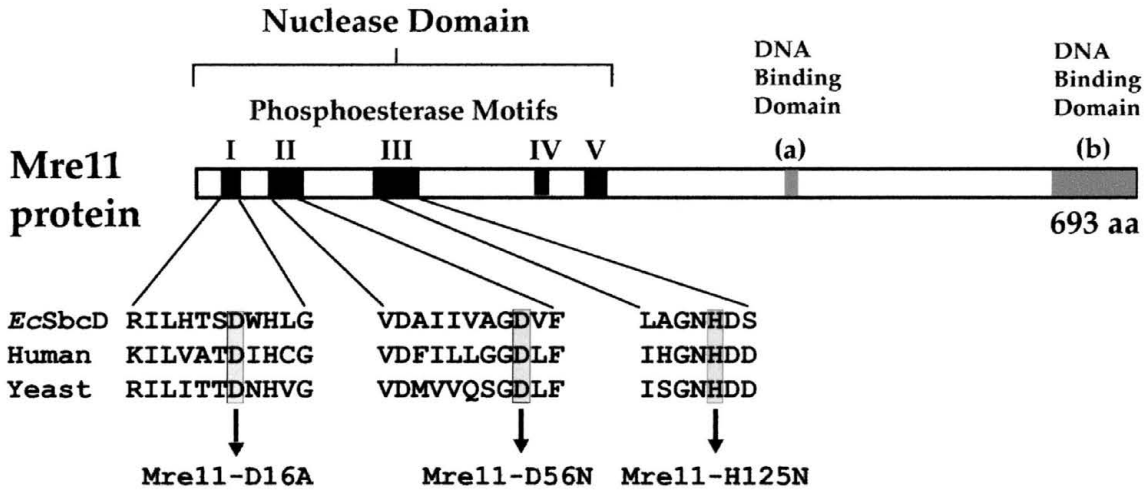


Figure 4. Three proposed nuclease deficient *mre11* mutants containing single amino acid substitutions within the conserved phosphoesterase motifs of the enzyme.

Previous studies with *mre11-D56N* and *mre11-H125N* indicated that these cells were mostly proficient in assays of DNA repair and DNA stability during normal mitotic growth (4). The conclusions drawn from these observations were surprising, stating that the major enzymatic function of RMX, ssDNA endo and dsDNA exonuclease activities were not essential for mitotic recombination, radiation resistance, or telomere stability. However, more recent work in our lab has shown that the third mutant, *mre11-D16A*, is completely defective in assays of mitotic recombination and is much more gamma sensitive than the other two mutants (Lewis, Storici, Calero and Resnick, submitted) (Figures 5-6).

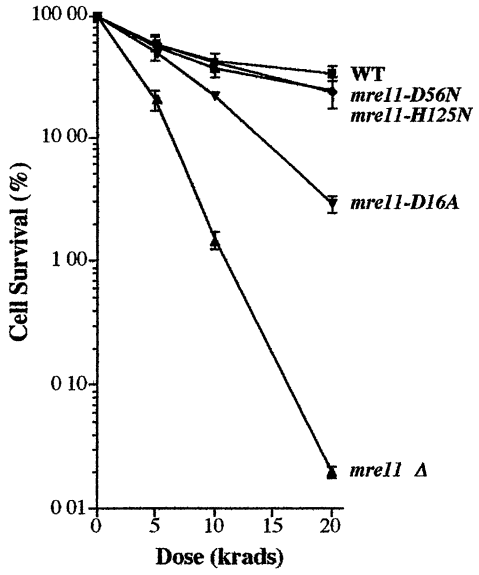


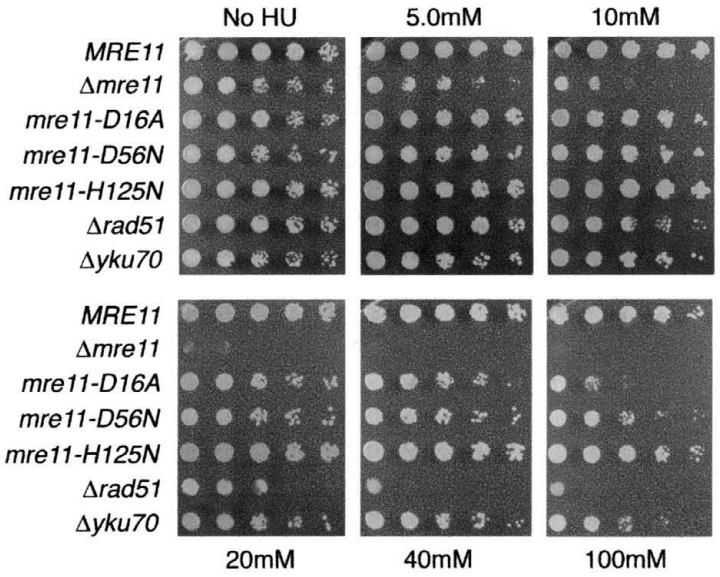
Figure 5. Gamma radiation survival curve of *mre11* nuclease mutants.

Cells lacking RMX are deficient in DSB repair and also in a process referred to as the S-phase checkpoint response to DNA damage. This means that RMX mutant cells damaged while in S phase (when DNA replication occurs) do not stop cycling in order to allow time for repair of the DNA damage. The uninterrupted replication of lesion-containing DNA in the mutants results in loss of viability.

Hydroxyurea (HU) is an inhibitor of the enzyme ribonucleotide reductase that, when expressed at high levels, leads to inhibition of DNA replication and formation of DSBs in chromosomal DNA (32, 33). Exposure to low levels of HU results in production of few DSBs, but results in activation of the S-phase checkpoint and killing of cells that are deficient in this checkpoint response. RMX mutants show reduced survival after exposure to low levels of HU. In addition, early checkpoint activation events such as phosphorylation of Rad53 are inhibited (33). To investigate the role of the nuclease activities of Mre11 in

the S-phase checkpoint, HU sensitivity was monitored in *mre11-D16A, mre11-D56N, mre11-H125N* mutants and in several control repair mutants (Figure 6A). HU survival assays were performed by dilution pronging and 5-fold dilutions of cells. The strains used for the assays were YLKL503 (*mre11*) containing pRS314 or *MRE11* plasmids. Control strains were YLKL532 (*Δrad51*) and YLKL593 (*Δyku70*) containing pRS314. Cells were grown on synthetic glucose plates with increasing concentrations of HU.

A.



B.

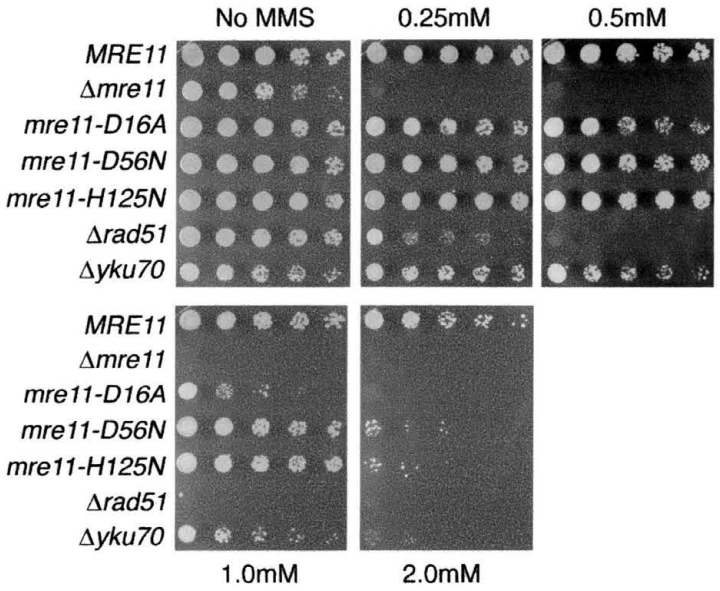


Figure 6. (A) Sensitivities of *mre11* nuclease mutants to hydroxyurea were compared to those of wildtype, *rad51* (Rec$^-$) and *yku70* (NHEJ$^-$) cells.

(B) Sensitivities of *mre11* nuclease mutants to methyl methanesulfonate were compared to those of wildtype, *rad51* (Rec$^-$) and *yku70* (NHEJ$^-$) cells.

mre11 strains began exhibiting growth inhibition at concentrations of HU as low as 5.0 mM. These cells were moderately more sensitive than Rec$^-$ *rad51* cells and much more sensitive than NHEJ-deficient *yku70* cells. Cells expressing the phosphoesterase mutants Mre11-D16A, -D56N and –H125N required much higher doses of HU to detect loss of viability than in *mre11* null cells. An important observation was that *mre11-D16A* strains exhibited killing at a lower dose (40mM) than either of the other nuclease mutants and was clearly more sensitive at 100 mM HU. The lack of sensitivity to lower doses of HU suggests that *mre11-D16A* mutants are not defective in checkpoint activation but have reduced repair of the DSBs produced by higher levels of HU. In addition, the greater sensitivity of *mre11-D16A* cells compared to the other two mutants is consistent with the radiation survival curves (Figure 5) and also to MMS survival assays that were performed (Figure 6B).

MMS is a DNA methylating chemical that, like HU, generates DNA DSBs during S-phase. The DNA strand breaks are thought to be produced by the processing of methylated bases by nucleases and subsequent inhibition of DNA replication (12). As with HU, RMX mutants are more MMS sensitive than other DNA repair mutants because of these S-phase defects. DNA repair mutants analyzed for HU sensitivity above were also assayed for MMS sensitivity (Figure 6B). Although all of the nuclease mutants were sensitive to MMS, once again *mre11-D16A* was clearly most sensitive (e.g. see 1 mM MMS plates in Figure 6B). In addition, none of the three nuclease mutants exhibited the extreme MMS sensitivity of *mre11* cells, further suggesting that checkpoint functions were not impaired.

Results of experiments performed here and in other labs are summarized in Table 3. As shown in the table, cells expressing *mre11-D16A* exhibit several dramatic mitotic DNA

repair defects that are more severe than those seen in the other phosphoesterase mutants with reduced *in vitro* nuclease activities. *mre11-D16A* is functional in protein:protein and protein:DNA association assays and is NHEJ-proficient *in vivo*, indicating that many normal functions are retained. We suggest that the strong radiation sensitivity and recombination defects are due primarily to lack of nuclease processing by the mutant Rad50/Mre11-D16A/Xrs2 complex *in vivo*. However, other explanations are possible. For example, the endo- and exonuclease activities, whose precise roles in DNA processing *in vivo* remain unclear, may be differentially affected in the mutants. It is also important to note that telomeres are shortened in *mre11-D16A* cells (Table 3). This result might also be due to a greater loss of nuclease activities in this mutant and supports the idea that chromosome ends (T-loops?) require processing by RMX, possibly to create substrates for DNA replication by telomerase (34). Mre11 nuclease function is required for RMX-mediated recombinational repair of DSBs in mitotic cells and that phenotypic differences between the mutants are likely to reflect differences in nuclease activities *in vivo*. In contrast, the nuclease function is not required for NHEJ repair.

TABLE 3. Impact of Mre11 proteins with reduced *in vitro* nuclease activities on DNA repair and stability [a]

Allele	In vitro Nuclease Activities		Associations			Plasmid	Spont. Diploid	Survival		DSB Repair By Recombination		Telomere	Meiotic
	Endo	Exo	DNA	Mre11	R/X[b]	NHEJ	Recomb.	Radiation	MMS	Ends-in	Ends-out	Stability	Recomb.
MRE11	+	+	+	+	+	+	+	+	+	+	+	+	+
mre11Δ	na	na	na	na	na	---	+++	---	---	---	---	---	---
mre11-D16A	---	---	+	+	+[c]	+	+	--	--	---	---	--	---
mre11-D56N	---	---	nd	nd	+[c]	+	+	+/-[d]	+/-[d]	-	nd	+	---
mre11-H125N	---	---	nd	nd	+	+	+	+/-[d]	+/-[d]	-	nd	+	---
mre11-H125L/	nd	nd	nd	nd	+	nd	+	+/-[d]	+	nd	nd	+	---

Symbols: na, not applicable; nd, no data; +, wildtype efficiency; +++, higher than wildtype; -, slight deficiency; --, moderate deficiency; ---, strong deficiency.

[a] References: Mre11-D56N and Mre11-H125N (MOREAU *et al.* 1999; SYMINGTON 2002; this work) ; Mre11-D16A (FURUSE *et al.* 1998; this work)

[b] R/X: Binding of mutant Mre11 protein to Rad50/Xrs2 using *in vitro* or *in vivo* assays.

[c] P. SUNG, personal communication.

[d] Mutants exhibit more killing than wildtype cells only at high doses of radiation (>30 krads) or high concentrations of MMS (this work; MOREAU *et al.* 1999; data not shown).

[e] Different levels of MMS-sensitivity were reported (TSUBOUCHI and OGAWA 1998; USUI *et al.* 1998; LEE *et al.* 2002).

Impact of varying cellular levels of telomerase components on DNA repair in RMX mutants.

Our laboratory has previously observed suppression of DNA repair deficiencies in yeast *rad50, mre11* and *xrs2* mutants when levels of *TLC1* RNA (the RNA subunit of telomerase) were increased, directly demonstrating that components of telomerase have an impact on DSB repair pathways (12). It follows then that one of the next inquiries would address whether other components of the telomerase complex can suppress killing of DSB repair mutants. The telomerase complex is composed of *TLC1* RNA plus several protein subunits. One subunit, Est2, possesses the polymerase activity of the enzyme complex. Other subunits include Est1, Est3 and Cdc13. In addition, Ten1 and Stn1 have been suggested to be members of the complex (14).

The effects of increasing levels of *EST2, TLC1* or *EXO1* on repair of *Eco*RI-induced DSBs in *mre11* cells were compared. A simple model was used to approach this issue and is shown schematically in Figure 7. High copy number plasmids containing *EST2, TLC1* or *EXO1* under the control of the strong constitutive *ADH1* promoter were transformed into *mre11* strain YLKL407 (*mre11*). This strain contains a mutation (*reg1-501*) that permits the modulated induction of *GAL* promoter activity driving expression of *Eco*RI endonuclease, while cells continue to grow in glucose (12, 35). *Eco*RI is a restriction enzyme (recognition sequence G^AATTC) that cleaves dsDNA to produce DSBs with 5' overhangs that are four bases in length (Figure 7). It has been demonstrated that *Eco*RI-induced DSBs cause the arrest of cell growth and slow loss in viability in *rad52* mutants that are recombination deficient. In contrast, cell killing is

much greater in *rad50*, *mre11* and *xrs2* mutants, which are defective in recombination and NHEJ (36).

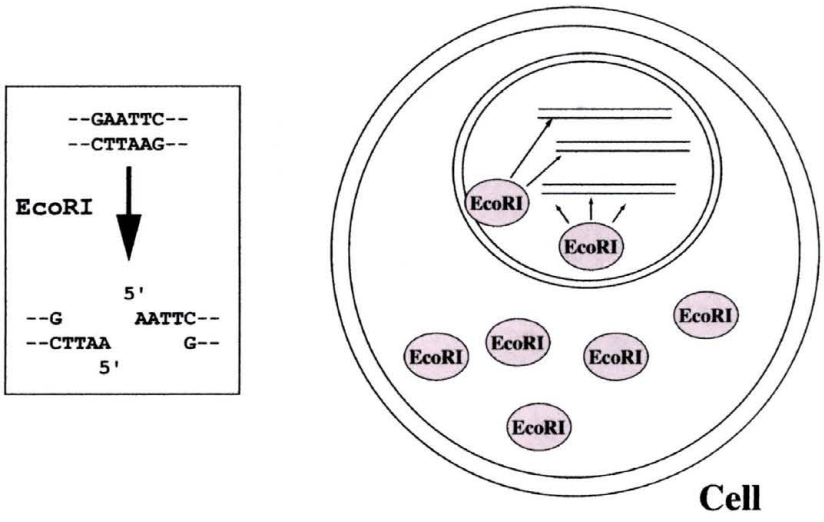


Figure 7. *In vivo* expression of *Eco*RI causes breaks in chromosomal DNA.

As shown in Figure 8 below, *mre11* cells containing only a cloning vector (pRS315URA) are killed after induction of *Eco*RI expression *in vivo* using 0.2% galactose. In this experiment, cells were grown overnight in liquid glucose media and then serially diluted 5-fold before pronging onto glucose (no *Eco*RI expression) or glucose plus galactose (inducing the *GAL1p::EcoRI* promoter fusion). Overexpression of Est2, however, suppresses the sensitivity of *mre11* cells to an extent that is comparable to the rescuing capabilities exhibited by Exo1 and *TLC1* RNA overexpression. Exo1 one is a 5'→3' exonuclease that has been previously implicated in recombination, mismatch repair and DNA replication. Studies have suggested that Exo1 nuclease activity may

substitute, although inefficiently, for the nuclease activity of the RMX complex thereby suppressing the lethality of these mutants when DNA DSBs are induced.

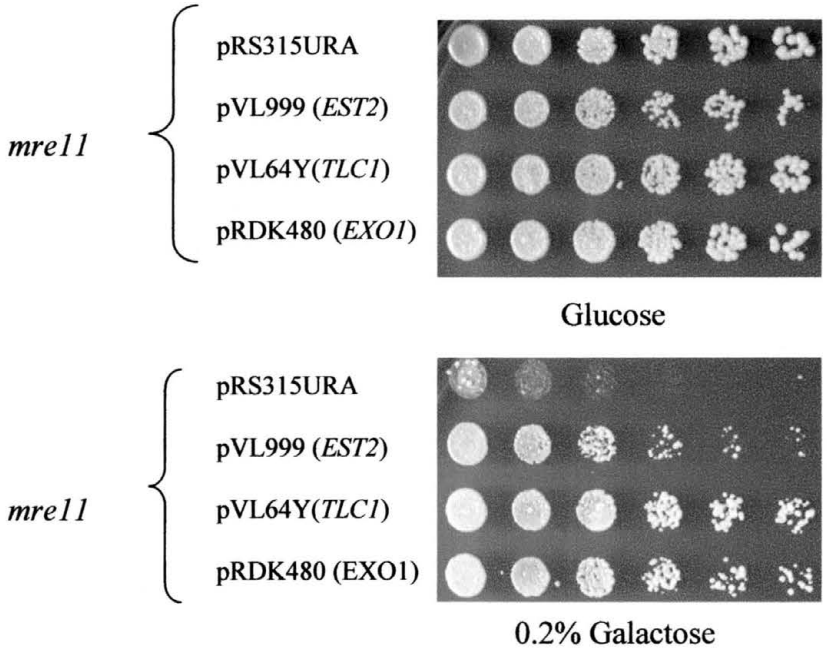


Figure 8. Expression of telomerase components Est2 and *TLC1* RNA, as well as Exo1 exonuclease, suppresses the lethality caused by expression of *Eco*RI in *mre11* mutants. *mre11* cells constitutively overexpressing Est2, *TLC1* RNA and Exo1 were transferred to 0.2% galactose plates to induce expression of *Eco*RI.

To follow up the previous result, other proposed components of the telomerase complex were tested. Plasmids containing genes encoding other telomerase components were transformed into *mre11* strain YLKL407. The cells were pronged onto media with (0.2%) and without galactose as in Figure 9. As seen in Figure 9, *Eco*RI expression was lethal in *mre11* cells and was rescued by *TLC1* RNA and Est2. However, overexpression of the Est1 subunit was ineffective (Figure 9).

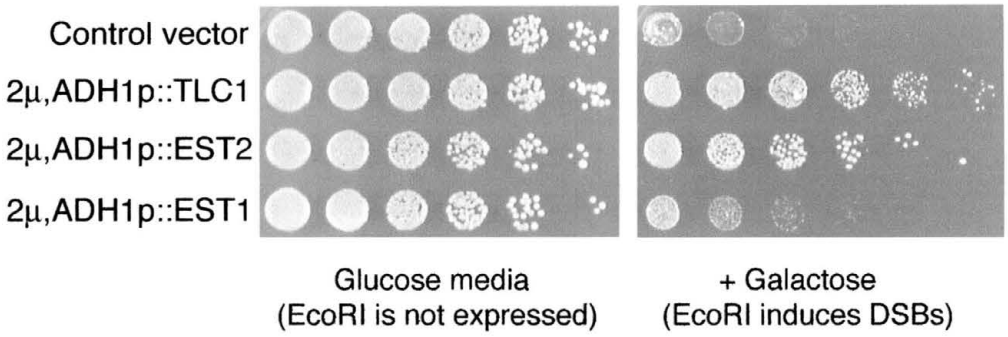


Figure 9. Overexpression of telomerase components Est2 (polymerase subunit) and TLC1 RNA (RNA template component) suppress killing by DNA damaged induced by *Eco*RI endonuclease. Telomerase component Est1 showed no effect in reducing lethality of *mre11* strains. High copy number plasmids expressing telomerase components were transformed into *mre11* mutant strainYLKL407. The cells were plated on galactose to induce damage by *Eco*RI.

The impacts of *CDC13, TEN1* and *STN1* on DSB repair in *mre11* mutants were also tested. As before, EcoRI expression was lethal in *mre11* cells. *EST2* and *TEN1* overexpression led to increased resistance to *Eco*RI (Figure 10). However, *STN1* did not rescue the lethality and *CDC13* overexpression produced only a weak response. Because of selection marker considerations (*URA3* versus *LEU2*), a different Est2 plasmid (pVL715) was used for this experiment than the one used for the previous experiment (pVL999) (Figure 10). Although survival was clearly increased by this construct, cell growth was not as rapid as with the other plasmid (pVL999). We presume that this is an indication that Est2 protein levels were not as high when pVL715 was used. To be thorough, a different *STN1* plasmid (pVL1035) was tested for increasing resistance to

MMS. The results were as before; *STN1* overexpression did not rescue MMS sensitivity of *mre11* cells (data not shown).

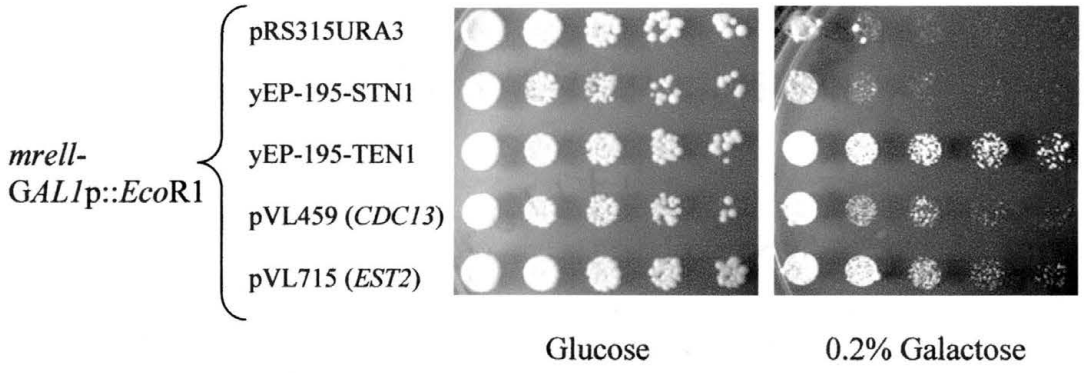


Figure 10. Repair of *Eco*RI-induced DSBs in *mre11* mutants is enhanced by elevated expression Ten1. *mre11* mutants overexpressing Stn1, Ten1, Cdc13, and Est2 were pronged onto 0.2% galactose plates to induce expression of *Eco*RI.

Our studies have revealed that several components of the telomerase complex, at increased intracellular levels, enhance the survival of *mre11* mutants, which are repair deficient and have shortened telomeres. An interesting question is whether wildtype cells, whose telomeres are normal length, experience the same enhancement in survival. Wildtype cells (YLKL350) were transformed with plasmids encoding Est2 or *TLC1* RNA. The cells were pronged onto glucose and glucose plus 0.2% galactose for the induction of *Eco*RI expression. Growth at 30°C for 2-3 days revealed that both *EST2* and *TLC1* RNA overexpression in wildtype cells makes them more resistant to *Eco*RI-induced damage (Figure 11).

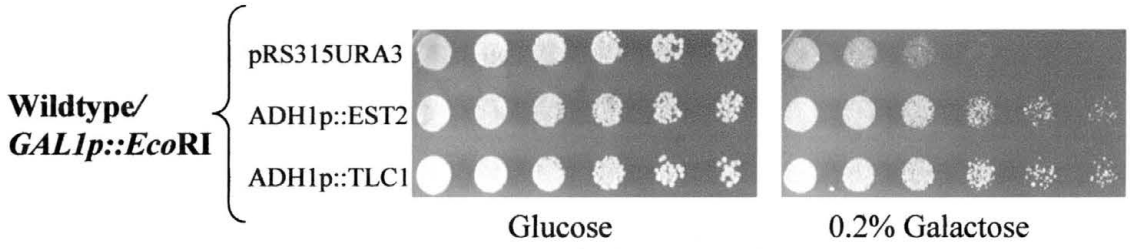


Figure 11. Overexpression of either *EST2* or *TLC1* RNA suppresses the modest *Eco*RI-induced killing and growth inhibition seen in wildtype cells (strain YLKL350). Five fold dilutions of wildtype cells overexpressing Est2 protein or *TLC1* RNA were transferred to plates containing galactose to induce expression of *Eco*RI endonuclease.

Evaluation of Est2 catalytic mutants

The Est2 subunit of the telomerase complex contains the polymerase activity and its reverse transcriptase motifs are essential for telomeric DNA synthesis (12). In work done by Lingner *et al.*(1997) to define the importance of the catalytic domain of the telomerase complex, mutant Est2 proteins containing substitutions in three conserved aspartates common to many reverse transcriptases (Asp→Ala) resulted in loss of normal telomerase activity shown schematically in Figure 12. Transformants expressing these mutants showed telomere shortening and cellular senescence.

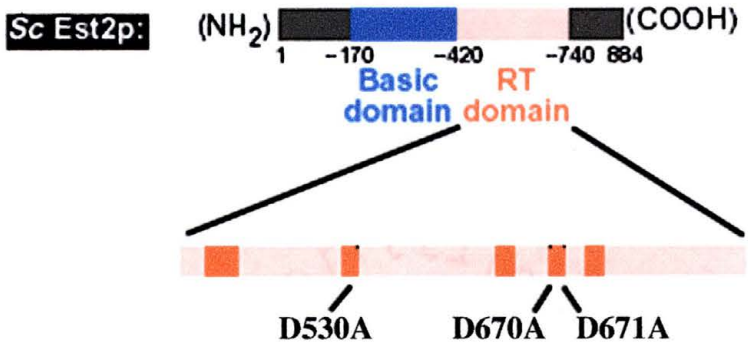


Figure 12. Catalytically inactive Est2 mutants containing Asp→Ala substitutions in the conserved reverse transcriptase domain of the enzyme.

We wanted to investigate the effects of expressing these stable but catalytically inactive mutants of Est2 in cells that were *mre11*-deleted in order to determine if the DNA synthesis function of the telomerase complex is required for repair. High copy plasmids containing three Est2 mutants under the control of the *ADH1* promoter (*est2-D530A, est2-D670A* and *est2-D671A*) were transformed into YLKL350 (*MRE11*$^{+}$) and *mre11* strain YLKL407. These strains contain a *GAL1p*::*EcoRI* integrated into chromosome XV. Constitutive overexpression of wildtype Est2 polymerase had no effect on *MRE11+* or *mre11* mutant cells when cells were grown in normal glucose media (i.e. under conditions where *Eco*RI endonuclease was not expressed) (Figure 13B). Surprisingly, expression of the catalytically inactive proteins did not affect growth of wildtype cells, but was toxic to *mre11* mutants even though *Eco*RI expression was not turned on (see results for *est2-D530A* and *est2-D670A* shown in Figure 13A and 13B.

Expression of the mutant proteins was also found to be lethal in *mre11* mutants of two other yeast strain backgrounds, VL6α and YPH102 (data not shown).

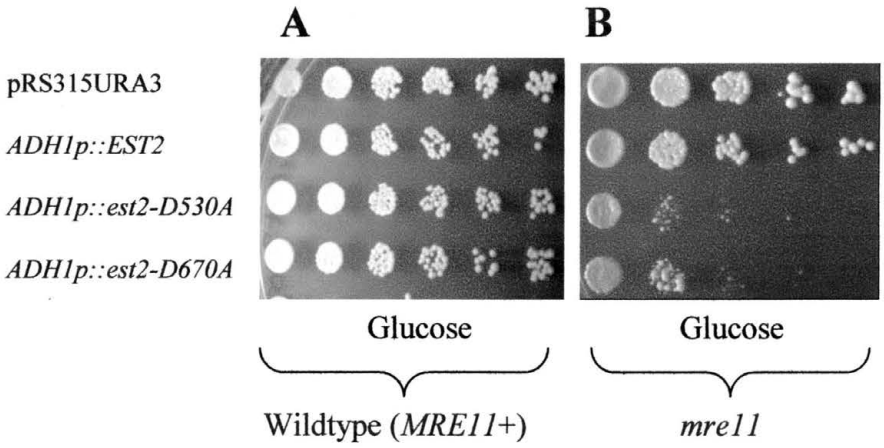


Figure 13. (A) Est2 mutants *est2-D530A* and *est2-D670A* were transformed into *MRE11* wildtype strain YLKL350. The cells exhibited normal growth on glucose media. (B) Est2 mutants *est2-D530A* and *est2-D670A* were transformed into *mre11* strain YLKL407. Overexpression of the mutant proteins proved to be toxic to this strain when grown on glucose even without induction of *Eco*RI.

Phenotypically, *mre11* cells have been shown to have shorter telomeres than wildtype cells (10). A possible explanation for the toxicity of the three Est2 mutants is that these catalytically inactive proteins form multi-subunit complexes *in vivo* that lack polymerase activity. The dominant negative effects may be the result of reduced DNA synthesis at the telomeres, thus causing the short telomeres of *mre11* mutants to become even shorter, eventually leading to senescence. This suggestion is supported by the normal growth seen when the mutant proteins were expressed in wildtype cells that have normal telomere lengths.

The impact of a representative Est2 mutant, *est2-D530A*, on cell viability and DNA damage resistance in a yeast background recently used for the Yeast Genome Deletion Project (involving construction of a set of ~5,000 yeast mutants with a single non-essential gene inactivated in each strain) was also examined (37). Interestingly, overexpression of this Est2 mutant in the background BY4742 was not lethal to *mre11* cells. However, the increased MMS sensitivity *est2-D530A* caused in *mre11*-BY4742 cells may be the consequence of a combination effect. DSBs are generated during processing and replication past methylated bases (primarily N7-meG and N3-meA) induced by MMS. Telomeres contain G-rich telomeric repeat sequences (TG_{1-3}) that may be preferential targets for MMS-induced damage compared to the rest of the chromosome. The combination of MMS damage at the telomeres, together with the dominant negative effect of expressing the Est2 mutant protein may result in the rapid reduction of telomeric ends, ultimately leading to rapid senescence. This idea, however, requires further investigation.

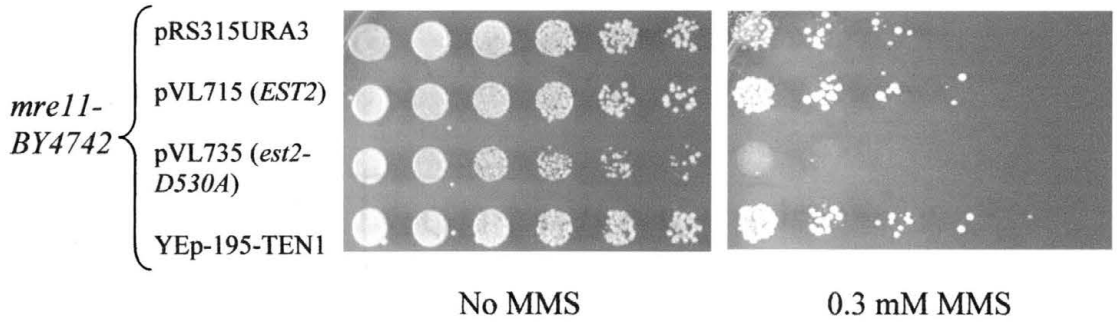


Figure 14. Expression of mutant protein containing a substitution within the active site of Est2 (*est2-D530A*) makes *mre11* cells of strain BY4742 more sensitive to MMS-

induced damage. Strains were grown on 2% Glu-Ura plates with and without 0.3 mM MMS.

Effects of Cdc13 end-binding protein overexpression on DSB repair

Cdc13 is a single-stranded DNA end-binding protein that may recruit the rest of the telomerase complex to chromosome ends. This protein binds preferentially to TG_{1-3} repeats within the telomere sequence and functions in both telomere end protection and telomere replication. This brought upon the notion that maybe Cdc13 can have a negative effect on repair by binding to the ends of a DSB, preventing the processing of the break by repair complexes. A possible explanation for enhancement of DNA repair in *mre11* mutants by *EST2* is that high levels of the protein lead to association with and titration of other telomerase components away from DSB ends. This recruitment away from the ends may then allow DNA repair enzymes access to the break thereby increasing DSB repair. If the end-binding telomerase protein encoded by *CDC13* is able to bind the DSB ends in the interior of the chromosome, then it may be the factor that is titrated away by Est2 (or *TLC1* RNA or Ten1). *dnl4* (NHEJ-deficient) and wildtype-BY4742 transformants expressing Cdc13 were exposed to 2 mM MMS or 200 mM HU to determine whether Cdc13 expression would be detrimental to DSB repair (Figure 15). *dnl4* mutants were included to preclude the possibility of residual repair by NHEJ and see effects specifically involving the recombination pathway. The results of this experiment showed no obvious increase in MMS or HU sensitivity caused by overexpression of Cdc13.

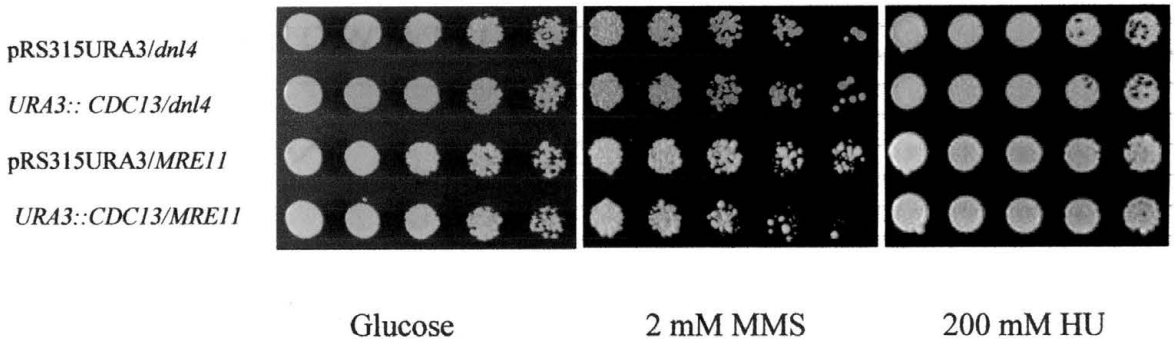


Figure 15. Overexpression of Cdc13 did not reduce DSB repair proficiency of *dnl4* or wildtype cells. Cdc13 was overexpressed in end-joining mutants (*dnl4*) and in wildtype cells. The cells were pronged onto media containing no clastogen, MMS or HU.

Southern blot analysis of telomere lengths in mre11 mutants overexpressing telomerase components.

Originally, three possible mechanisms by which overexpression of telomerase components (*TLC1* RNA, *EST2* and *TEN1*) increased resistance to chemical and physical clastogens were proposed. The first model suggests that overexpression of telomerase components results in an increase in recombinational repair. Related, is the idea that this increased resistance results from an enhancement in end-joining repair (NHEJ). Lastly, it may also be possible that the sites of DSBs are stabilized by *de novo* telomere addition thereby stabilizing broken chromosomes. Similar to the *de novo* telomere addition model, which proposes that new telomere ends added to the site of DSBs results in the stabilization of the chromosome allowing the cell to survive more replications before

entering crisis and ending in senescence, is the idea that telomerase components may be working to stabilize the actual telomeres of *mre11* cells. A way this may be possible is if these telomerase components are stabilizing *mre11* mutant telomeres by actually adding length to them. Therefore, an important question is whether overexpression of *TLC1*, Est2 or Ten1 affects the already shortened telomere lengths of *mre11* cells. To address this possibility a non-radioactive Southern blot analysis of telomere lengths in *mre11* mutants overexpressing telomerase components was performed.

The entire procedure is described in detail in chapter two. DNA from YLKL568 (wildtype strain BY4742), YLKL650 (*mre11*-BY4742), and YLKL 650 transformed with pRS315URA3 (vector), pVL999 (*EST1*), pVL64Y (*TLC1*), pRDK480 (*EXO1*) and Yep-195-TEN1 was purified using an Epicenter MasterPure™ Purification Kit. The DNA was digested with *Xho1* restriction enzyme, separated by electrophoresis through a 1.2% agarose gel (1μg sample/well), and then blotted onto N^+ Hybond membrane via capillary transfer. The membrane was probed with a DNA fragment that had been labeled with digoxigenin-dUTP (DIG) (Figure 16A). The fragment was created by incorporation of DIG-dUTP and amplification of a telomeric repeat containing plasmid YTCA-1 via polymerase chain reaction (PCR). The DIG-labeled DNA anneals to complementary sequences in the digested DNA bound to the membrane. Anti-DIG antibody (linked to alkaline phosphatase) was added to the membrane which was then washed to avoid non-specific binding. The reaction was initiated by the addition of CSPD substrate. When alkaline phosphatase cleaves CSPD it results in a chemiluminescent signal (Figure 16B). An overview of the entire process is shown schematically in Figure 17. Visualization of the blot was achieved by exposing X-ray film.

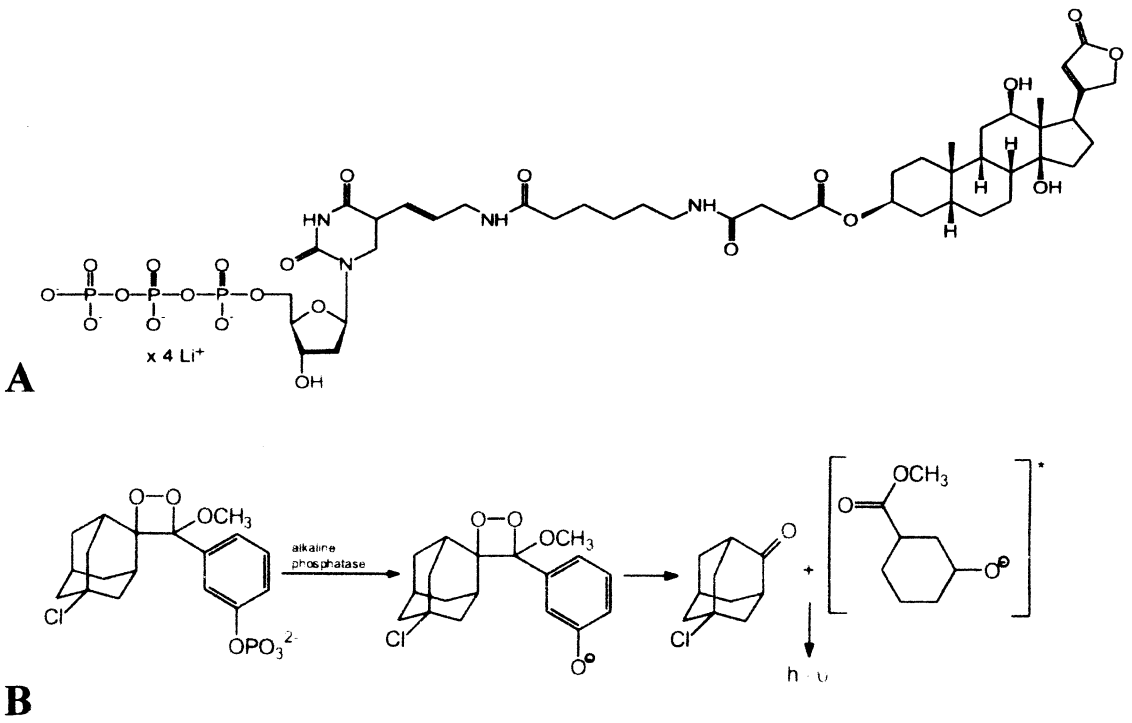


Figure 16. (A) Chemical structure of digoxigenin. (B) Reaction showing cleavage of CSPD by alkaline phophatase.

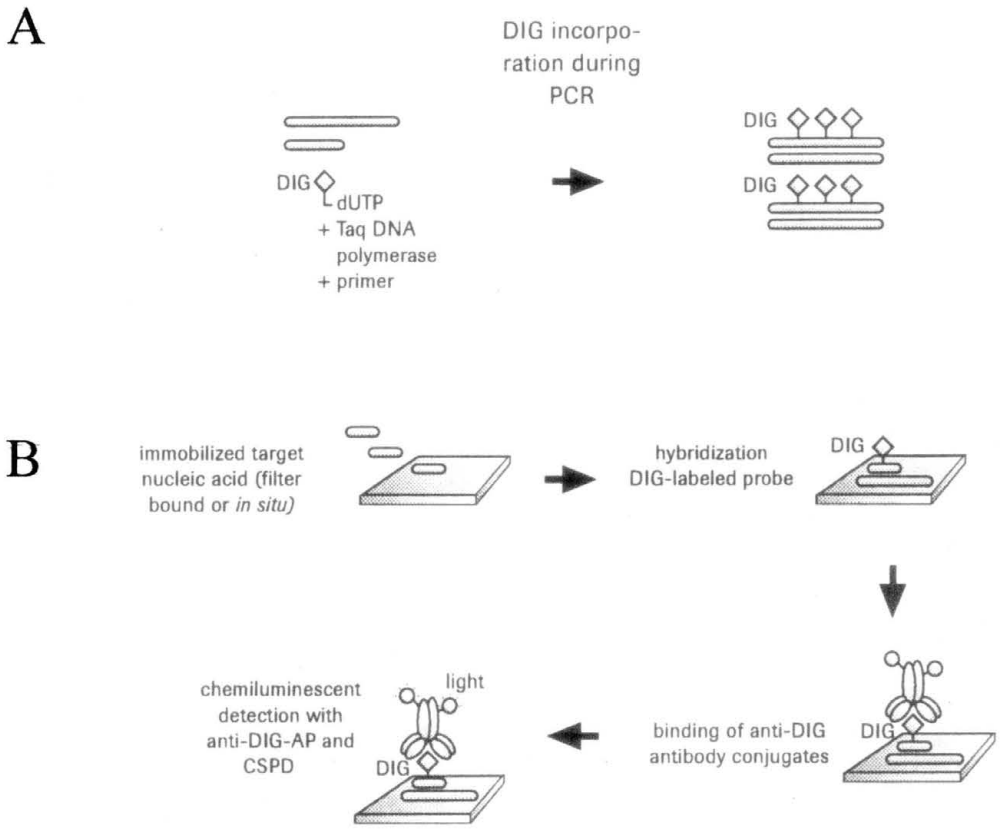


Figure 17. Protocol for non-radioactive labeling and detection for Southern blotting using digoxigenin. (A) Preparation of DIG-labeled probe DNA by PCR. (B) Outline of DNA transfer, hybridization and chemiluminescent detection procedures.

As there is some variability among different yeast strains, a test was performed to confirm that *mre11* strain BY4742 was phenotypically consistent with observations made of other yeast strains, where *mre11* mutants have shorter telomeres compared to wildtype. Figure 18 shows that this was indeed the case. *Xho*I cuts ~1200 bp from the ends on some, but not all yeast chromosomes. Thus, wildtype cells typically produce a broad ~1200 bp band when probed with a telomeric DNA fragment. The wildtype telomere band in BY4742 DNA migrated to 1200bp, while the *mre11* telomere band migrated lower at ~1000 bp (Figure 18). Both bands were quite broad, an indication that, as seen before (11, 30) telomeric endpoints were highly variable. In addition to this lower band, all other DNA fragments from mre11 cells were smaller than their counterparts in wildtype cells.

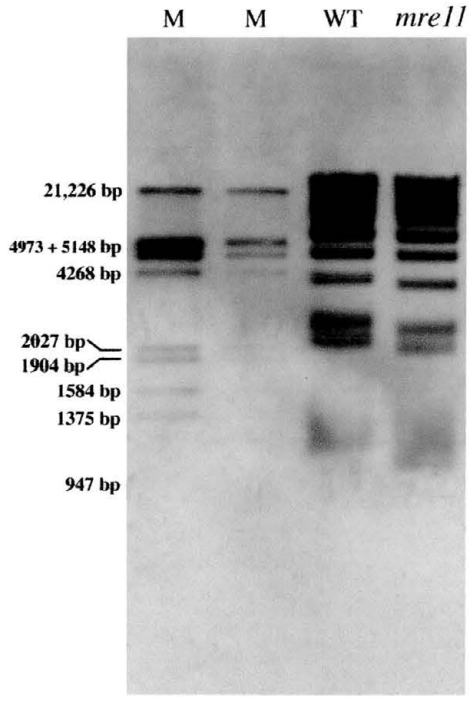


Figure 18. Southern analysis was used to observe the phenotypic shortening of telomeres in mre11 mutants. Genomic DNA from YLKL568 (wildtype-BY4742) and YLKL650 (*mre11*) was purified and digested with *XhoI*, separated by

electrophoresis through a 1.2% agarose gel, and blotted onto a nylon membrane. The membrane was probed with telomeric repeat sequences derived from the plasmid YTCA-1.

Once it was established that *mre11*-BY4742 did show shortening of telomeres similar to other strain backgrounds, the effects of overexpressing telomerase components in this strain were examined. In this experiment, wildtype and *mre11*-BY4742 DNA were run as positive controls. As described earlier, Exo1 exonuclease overexpression in *mre11* mutants makes cell more resistant to various physical and chemical clastogens. In a studies by Moreau *et al.* (2001)(38) and Lee *et al.* (2002), Exo1 overexpression did not show any effect on the length of *mre11* telomere ends. Therefore, Exo1 was used as a negative control. The Southern blot analysis in Figure 19 shows again, that *mre11* mutants have shorter telomeres than wildtype cells, and that Exo1 overexpression does not affect telomere lengths. Interestingly, none of the three telomerase components (Est2, *TLC1* RNA, or Ten1), which all showed enhancement of survival of *mre11* mutants after exposure to DNA-damaging agents, had any effect on the lengths of telomeres.

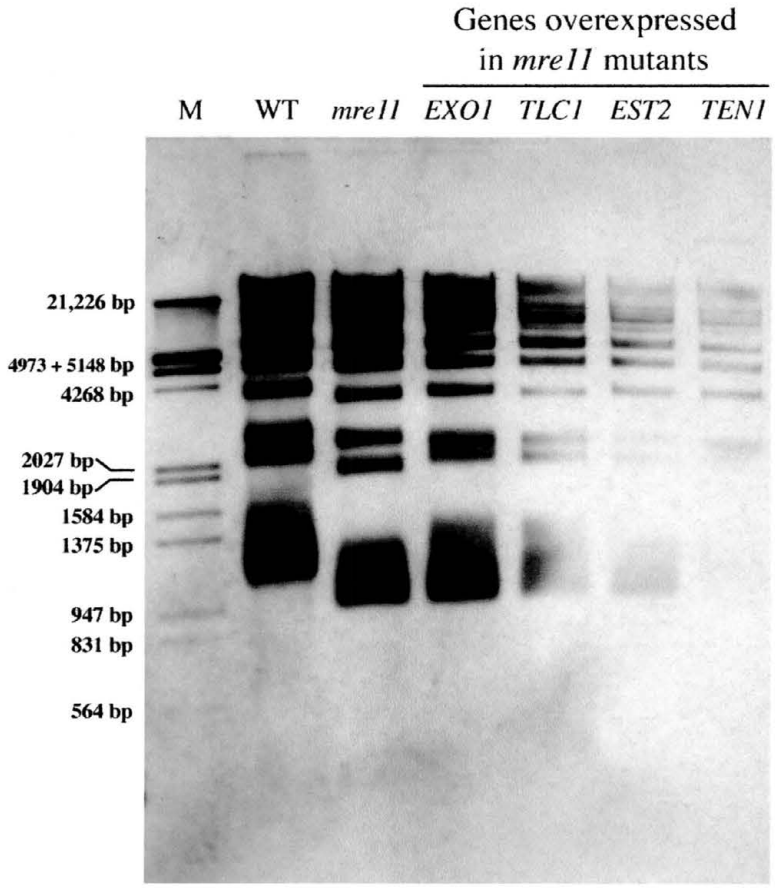


Figure 19. Southern analysis of mre11 mutants overexpressing Est2, TLC1 RNA, or Ten1 showed no telomere lengthening by the telomerase components. The procedure as described above was followed.

In summary, we have found first that the *mre11-D16A* nuclease mutant, which retains no nuclease function *in vitro*, but retains the ability to bind DNA and form stable RMX complexes, exhibits several detrimental mitotic DNA repair defects. The studies of HU and MMS sensitivity done as part of this thesis project plus previous results of studies done with *mre11-D16A* in our lab argue that its defects are due to lack of nuclease activity, thereby suggesting that the nuclease function is indeed essential to the function

of the complex in DSB repair. This is in contrast to other previously characterized mutants (D56N and H125N), whose phenotypes in DNA repair assays have resulted in the conclusion that the nuclease function of the RMX complex is not essential for its role in DSB repair. Of great relevance to the second part of this project is the past observation that telomeres in *mre11-D16A* mutants are shortened (though not in *mre11-D56N* or *mre11–H125* mutants), which suggests that the nuclease activity of RMX is required for telomere maintenance. This raises the possibility is that the chromosome ends (possibly T-loops) require processing by the RMX nuclease complex, perhaps to create substrates for DNA replication by the telomerase complex (34).

Following the previous observation in this lab that overexpression of *TLC1* (the RNA template subunit of the telomerase complex) increased survival of DSB repair mutants, we found that other subunits, Est2 (polymerase subunit) and Ten1 also enhanced repair. Telomerase subunits Stn1, Cdc13, and Est1 had no effect on survival of DSB repair mutants. In analyses done to determine whether the catalytic activity of Est2 was required for the enhanced survival in *mre11* cells, it was found that overexpression of enzymatically inactive Est2 mutants was toxic in *mre11* strains of some yeast backgrounds. We suggest that the dominant negative effects demonstrated by these mutants may be the result of reduced DNA synthesis at the telomeres, thus causing the short telomeres of *mre11* mutants to become even shorter, eventually leading to senescence. Another *mre11* strain background showed increased sensitivity to MMS when a catalytically inactive Est2 protein was overexpessed. Our position on this finding is that the combination of MMS damage at the telomeres, together with the dominant

negative effect of expressing the Est2 polymerase defective protein, may result in the rapid reduction of telomeric ends, ultimately leading to senescence.

Based on the previous characterization of the function of Cdc13 as a single-stranded DNA end-binding protein that may recruit the rest of the telomerase complex to chromosome ends tests were done to determine if overexpression of Cdc13 would negatively affect DNA DSB repair. Our hypothesis was that Cdc13 can have a negative effect on repair by binding to the ends of a DSB, preventing the processing of the break by repair complexes. Our results indicate that this is probably not the case as overexpression of Cdc13 showed no obvious effect on MMS or HU sensitivity.

Finally, we determined by Southern blot analysis that the Est2, *TLC1* RNA and Ten1 subunits of telomerase did not contribute to the increased viability of the cells by adding telomere length to the shortened ends of *mre11* cells. This observation narrows down the list of proposed mechanisms by which overexpression of the telomerase complex is working to increase survival of DSB repair mutants, which include an increase in recombination activity or NHEJ activity, stabilization of DSB ends by *de novo* telomere addition, and stabilization of the telomeres by end replication.

In conclusion, results obtained in this work have increased our understanding of the repair processes mediated by the Rad50/Mre11/Xrs2 complex. Furthermore, they have provided evidence that intracellular levels of several telomerase components can determine the level of DNA repair in repair-deficient cells and, surprisingly, even in wildtype cells. The importance of these observations is clear when considering the many diseases that result from deficiencies in DNA repair. Possibly of even greater importance, they suggest that the resistance of cancer cells to the different kinds of DNA damaging

agents used in radiation therapy and chemotherapy is determined, at least in part, by the levels of telomerase RNA and protein expressed in the cells. With further investigation of these phenomena, it may be possible to design improved therapies involving targeting of telomerase along with conventional treatments to achieve better clinical results.

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