Telomere Dysfunction Drives Increased Mutation by Error-Prone Polymerases

Rev1 and ζ in Saccharomyces cerevisiae.

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Running Head: Telomere Dysfunction Drives Mutation

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Using a model system, we have shown that replicative senescence is accompanied by a 16-fold increase in base substitution and frameshift mutations near a chromosome end. The increase was dependent on error-prone polymerases required for the mutagenic response to DNA lesions that block the replication fork.
*S. cerevisiae* cells lacking telomerase, a ribonucleoprotein complex required for telomere replication, experience progressive telomere degradation that culminates in replicative senescence (Lendvay *et al.* 1996). Deletions that encompass the *CAN1* locus located approximately 32 kb from the telomere on the left arm of chromosome V accumulated during senescence (Hackett and Greider 2003; Hackett *et al.* 2001), and have been attributed to replication fork stalling (Motegi *et al.* 2006). Since replication fork stalling also generates mutations (Quah *et al.* 1980), we investigated the effect of telomere dysfunction on the generation of mutations at the *CAN1* locus.

**Mutation Rate Analysis:**

We examined the behavior of mutants defective for *EST2*, which encodes the catalytic subunit of telomerase (Counter *et al.* 1997). We determined the mutation rate at the *CAN1* locus using an assay that selects against deletions in serial cultures of wild type and *est2* mutant cells. We observed no significant change from the wild type rate of *CAN1* mutation in *est2* cultures before senescence (*p* = 0.07), a 16-fold increase during senescence (*p* < 0.0001) and the restoration of wild type levels upon recovery (*p* = 0.09) (Fig. 1a). No senescence-dependent changes in the mutation rate at the *CYH2* locus, located 310 kb from its telomere on the left arm of chromosome VII, were observed in *est2* mutant cultures before (*p* = 0.68), during (*p* = 1.0), or after replicative senescence (*p* = 1.0) (Fig. 2), suggesting that the mutagenic effect is restricted to telomere proximal sequences.

The *can1* mutation spectrum observed for senescent *est2* mutant cells was similar to that of wild type (Table 2), suggesting that the mechanism of senescence-dependent
Mutagenesis in *est2* cells may be similar to the mechanism of spontaneous mutagenesis in wild type cells. Since 50-70% of spontaneous mutagenesis has been attributed to the action of error-prone polymerases (Quah *et al.* 1980), we investigated whether they were involved in the mechanism underlying senescence-dependent mutagenesis. We examined the effects of mutations in the *RAD30, REV7* and *REV1* genes, which are required for error-prone polymerase function in yeast (Goodman 2002; Johnson *et al.* 2000; Haracska *et al.* 2000; Prakash *et al.* 2005). Mutation rates in *est2 rad30* cultures before (p = 0.32), during (p = 0.34) and after senescence (p = 0.15) were not significantly different than in *est2* cultures (Fig. 1a, 1b), suggesting that Pol η does not contribute to senescence-dependent mutagenesis. In contrast the *rev1* and *rev7* mutations completely suppressed senescence-dependent mutagenesis, as the *CAN1* mutation rates were not significantly different from *rev1* and *rev7* mutants before (p = 0.46 *rev1*, p = 0.53 *rev7*), during (p = 0.84 *rev1*, p = 0.09 *rev7*) or after senescence (p = 1.0 *rev1*, p = 0.2 *rev7*) (Fig. 1a, 1b).

Southern blot analysis of representative canavanine resistant mutants collected from senescent cells revealed that all had unrearranged *can1* loci, consistent with base substitution and frameshift mutations (DM and AB, unpublished data). These data suggest that Rev1 and DNA polymerase ζ are required for senescence-dependent mutagenesis, perhaps through mutagenic bypass of DNA replication lesions generated during replicative senescence. The minimal effects of the *rad30, rev1* and *rev7* mutations on the growth, senescence and recovery of *est2 rad30, est2 rev1*, and *est2 rev7* cells (Fig. 3a, 3b), suggest that the mutation of telomere-proximal sequences does not contribute to the initiation of, or recovery from senescence.
Exo1 has been suggested to be necessary for destabilizing the CAN1 locus during senescence by promoting exonucleolytic degradation from the telomere (Hackett and Greider 2003). We observed no significant change in the rates of CAN1 mutation in est2 exo1 cultures before (p = 0.89) or after senescence (p = 1.0) from that observed in exo1 cultures, but did see a significant 10-fold increase during senescence (p < 0.0001)(Fig 1a, 1b). These data suggest that Exo1-dependent nucleolytic degradation is not required to observe senescence-dependent increases in CAN1 mutation rate.

**GCR Analysis:**

In addition to mutagenesis, we also examined the rate of gross chromosomal rearrangement (GCR) in est2 mutants grown serially over time. GCR is defined as an event that leads to the simultaneous loss of CAN1 and a URA3 marker inserted at the HXT13 locus that lies between CAN1 and the telomere on the left end of chromosome V (Chen and Kolodner 1999). The GCR rate was only two-fold over wild type before senescence, 383-fold over wild type during senescence, and decreased to wild type levels upon recovery from senescence (Table 3), all consistent with previously published reports (Myung et al. 2001; Pennaneach and Kolodner 2004; Hackett et al. 2001).

Consistent with CAN1 mutagenesis REV1 and REV7 were found to be important in determining the GCR rate during replicative senescence. GCR rates in est2 rev1 and est2 rev7 mutants were only 2-4 fold higher during senescence than in rev1 and rev7 mutants (Table 3) that did not undergo senescence (Fig. 3a). These results are consistent with Pol ζ and Rev1 being required for senescence-dependent GCR. Strikingly, Rad30 was found to be required to suppress GCR as the rate in est2 rad30 cells was 37-fold over wild type before senescence, 2,673 fold over wild type during senescence and at wild
type levels upon recovery (Table 3). Interestingly, this increase is nearly completely
dependent on Rev7 as the GCR rate in senescent est2 rad30 rev7 cells was less than 2-
fold greater than in the est2 rev7 mutant (Table 3). Therefore, like senescence-dependent
mutagenesis at the CAN1 locus, senescence-dependent GCR requires Rev1 and Pol ζ.

Our data suggest that Rev1- and Pol ζ-dependent mutations and GCR at telomere
proximal loci are an important consequence of telomere dysfunction. The involvement of
Pol η, Polζ and Rev1 suggests that the post-replication repair machinery (Minesinger and
Jinks-Robertson 2005) may be responding to the failure of bidirectional DNA replication
in the region (Lehmann 2005; Plosky and Woodgate 2004). Strathern and colleagues
have reported the involvement of Pol ζ in generating mutations associated with the
recombinational repair of an enzyme-catalyzed double-strand break (Rattray et al. 2002),
suggesting that a similar process may be involved in generating mutations at sequences
lying near the uncapped telomeres of senescent cells (Dubois et al. 2002). However,
such a mechanism is unlikely to involve extensive exonucleolytic degradation from the
telomere as loss of Exo1, a factor involved in the degradation of uncapped telomeres
(Hackett and Greider 2003), does not reduce senescence-dependent mutation (Fig. 1B) or
GCR (Table 3). Further, this mechanism would likely require homologous
recombination between sister-chromatids, as the CAN1 gene is a unique sequence in the
genome. Perhaps, similar forces underlie some of the increased genome instability and
cancer in somatic cells of elderly people (Lengauer et al. 1998; Johnson et al. 1999).
ACKNOWLEDGEMENTS

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LITERATURE CITED


Motegi, A., K. Kuntz, A. Majeed, S. Smith and K. Myung, 2006 Regulation of gross


Table 1: *S. cerevisiae* Strains Used in this Study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
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<tbody>
<tr>
<td>ABX1269</td>
<td>MATα ade2-1/ade2-1 CAN1/can1-100 HIS3/his3-11, 15 leu2-3, 112/leu2-3, 112 trp1-1/trp1-1 ura3::TRP1/ura3::TRP1 HXT13/hxt13::URA3 EST2/est2::LEU2 EXO1/exo1::hisG RAD5/RAD5</td>
</tr>
<tr>
<td>ABX1429</td>
<td>MATα ade2-101/ade2-1 can1-100/can1-100 his3::Δ200/his3-11, 15 leu2-Δ1/leu2-3, 112 trp1-1/trp1-1 ura3-52/ura3-1 adh4::URA3-TEL/ADH4 CYH2/CYH2 EST2/est2::LEU2 RAD5/RAD5</td>
</tr>
<tr>
<td>ABX1727</td>
<td>MATα ade2-1/ade2-1 CAN1/CAN1 HIS3/his3-11, 15 leu2-3, 112/leu2-3, 112 trp1-1/trp1-1 ura3::TRP1/ura3::TRP1 hxt13::URA3/hxt13::URA3 EST2/est2::LEU2 REV1/rev1::HIS3 RAD5/RAD5</td>
</tr>
<tr>
<td>ABX1729</td>
<td>MATα ade2-1/ade2-1 CAN1/CAN1 his3-31, 15/his3-11, 15 leu2-3, 112/leu2-3, 112 trp1-1/trp1-1 ura3::TRP1/ura3::TRP1 hxt13::URA3/hxt13::URA3 EST2/est2::LEU2 RAD30/rad30::HIS3 REV7/rev7::hisG RAD5/RAD5</td>
</tr>
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Table 2: CAN1 Mutation Spectra in Wild type and Mutant Cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Base Substitution</th>
<th>Frame-shift</th>
<th>Insertion/Deletion</th>
<th>Complex</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>50%</td>
<td>43.7%</td>
<td>0%</td>
<td>6.25%</td>
</tr>
<tr>
<td>est2</td>
<td>50%</td>
<td>50%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

* Genomic DNA was extracted from 32 independent can1 mutant colonies derived from wild type cells and 22 independent can1 mutant colonies derived from senescent est2 mutant cells and used to program PCR reactions to amplify the 2 kb CAN1 sequence using primers 101 (5’CTC GAG TTT ACG TAT ATA TCT GGA ACA G) and 102 (5’CTC GAG GGG TGA GAA TGC GAA ATG GCG). PCR products were purified and subjected to sequencing using primers 201 (5’TAT TGG TAT GAT TGC CCT TG), 202 (5’GAG TTC TGG GTC GCT TCC ATC), 203 (5’CAA TCT ACT TCC TAC GTT TC), 204 (5’GAA TAT GCC AAA GAA CCC) and 205 (5’GAG GGT GAG AAT GCG AAA T).
Table 3: Control of Senescence-Dependent Gross Chromosomal Rearrangement

<table>
<thead>
<tr>
<th>Genotype</th>
<th>GCR Rate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>25 generations&lt;sup&gt;b&lt;/sup&gt;</th>
<th>50 generations&lt;sup&gt;c&lt;/sup&gt;</th>
<th>150 generations&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td>1.73 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>1.07 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>1.98 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>est2</td>
<td></td>
<td>4.36 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>4.1 x 10&lt;sup&gt;-7&lt;/sup&gt; (383)</td>
<td>4.93 x 10&lt;sup&gt;-9&lt;/sup&gt; (2.5)</td>
</tr>
<tr>
<td>exo1</td>
<td></td>
<td>1.74 x 10&lt;sup&gt;-8&lt;/sup&gt; (10)</td>
<td>4.73 x 10&lt;sup&gt;-9&lt;/sup&gt; (4.5)</td>
<td>7.5 x 10&lt;sup&gt;-9&lt;/sup&gt; (4)</td>
</tr>
<tr>
<td>rad30</td>
<td></td>
<td>3.4 x 10&lt;sup&gt;-9&lt;/sup&gt; (2)</td>
<td>2.56 x 10&lt;sup&gt;-9&lt;/sup&gt; (2)</td>
<td>2.11 x 10&lt;sup&gt;-9&lt;/sup&gt; (1)</td>
</tr>
<tr>
<td>rev1</td>
<td></td>
<td>1.74 x 10&lt;sup&gt;-9&lt;/sup&gt; (10)</td>
<td>1.45 x 10&lt;sup&gt;-9&lt;/sup&gt; (13)</td>
<td>7.5 x 10&lt;sup&gt;-9&lt;/sup&gt; (4)</td>
</tr>
<tr>
<td>rev7</td>
<td></td>
<td>1.01 x 10&lt;sup&gt;-9&lt;/sup&gt; (6)</td>
<td>9.65 x 10&lt;sup&gt;-9&lt;/sup&gt; (9)</td>
<td>7.56 x 10&lt;sup&gt;-9&lt;/sup&gt; (4)</td>
</tr>
<tr>
<td>est2 exo1</td>
<td></td>
<td>1.2 x 10&lt;sup&gt;-8&lt;/sup&gt; (7)</td>
<td>3.19 x 10&lt;sup&gt;-7&lt;/sup&gt; (298)</td>
<td>1.31 x 10&lt;sup&gt;-8&lt;/sup&gt; (6.5)</td>
</tr>
<tr>
<td>est2 rad30</td>
<td></td>
<td>6.39 x 10&lt;sup&gt;-9&lt;/sup&gt; (37)</td>
<td>2.86 x 10&lt;sup&gt;-6&lt;/sup&gt; (2,673)</td>
<td>4.55 x 10&lt;sup&gt;-9&lt;/sup&gt; (2)</td>
</tr>
<tr>
<td>est2 rev1</td>
<td></td>
<td>8.84 x 10&lt;sup&gt;-9&lt;/sup&gt; (5)</td>
<td>3.1 x 10&lt;sup&gt;-8&lt;/sup&gt; (29)</td>
<td>1.53 x 10&lt;sup&gt;-8&lt;/sup&gt; (8)</td>
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<tr>
<td>est2 rev7</td>
<td></td>
<td>1.24 x 10&lt;sup&gt;-8&lt;/sup&gt; (7)</td>
<td>4.0 x 10&lt;sup&gt;-8&lt;/sup&gt; (37)</td>
<td>1.6 x 10&lt;sup&gt;-8&lt;/sup&gt; (8)</td>
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<tr>
<td>rad30 rev7</td>
<td></td>
<td>4.1 x 10&lt;sup&gt;-9&lt;/sup&gt; (2)</td>
<td>3.9 x 10&lt;sup&gt;-9&lt;/sup&gt; (3.5)</td>
<td>2.4 x 10&lt;sup&gt;-9&lt;/sup&gt; (1)</td>
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<tr>
<td>est2 rad30 rev7</td>
<td></td>
<td>4.7 x 10&lt;sup&gt;-8&lt;/sup&gt; (27)</td>
<td>6.95 x 10&lt;sup&gt;-8&lt;/sup&gt; (65)</td>
<td>5.79 x 10&lt;sup&gt;-9&lt;/sup&gt; (3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fresh spore colonies of the appropriate genotype were taken in their entirety from the dissection plate and dispersed in water. Aliquots were removed to determine viability following dilution, plating on to YPD medium and incubation at 30° for three days. The remainder was plated on to synthetic medium containing canavanine and allowed to form colonies at 30°C, at which point colonies were counted and replica plated on to synthetic medium lacking uracil. GCRs were defined as the canavanine resistant cells that had lost the ability to grow without uracil. The deletion rate was determined by fluctuation
analysis (Luria and Delbrück 1943). Five subsequent serial calculations of the GCR rate were performed using single colonies arising on the YPD viability plates.

\[ ^b \text{Deletion rate before replicative senescence, } \sim 25 \text{ generations.} \]

\[ ^c \text{Deletion rate during replicative senescence, } \sim 50 \text{ generations.} \]

\[ ^d \text{Deletion rate after recovery from replicative senescence, } \sim 150 \text{ generations.} \]

\[ ^e \text{Fold differences from wild type level at a comparable number of generations in parentheses.} \]
FIGURE LEGENDS

Figure 1. *REV1* and *REV7*, but not *RAD30* are required to observe increases in *CAN1* mutation rate during replicative senescence in *est2* mutant cells. a. *CAN1* mutation rate of ▲ wild type, △ *est2Δ*, ■ *rev1Δ*, ● *rev7Δ*, ▼ *rad30Δ* and ♦ *exo1Δ* mutant cells at the indicated time points. b. *CAN1* mutation rate of □ *est2Δ rev1Δ*, ○ *est2Δ rev7Δ*, ▼ *est2Δ rad30Δ*, ♦ *est2Δ exo1Δ*, × *rad30Δ rev7Δ* and ★ *est2Δ rad30Δ rev7Δ* mutant cells at the indicated time points. Spore colonies of the appropriate genotype were obtained from freshly dissected tetrads of ABX1269, ABX1727 and ABX1729 (Table 1) and dispersed in water. Aliquots were removed to determine viability following dilution, plating on to YPD medium and incubation for three days at 30°. The remainder was plated on to synthetic medium lacking arginine and containing 60 µg/ml canavanine, and incubated for 3 days at 30°. The colonies arising on the canavanine plates were counted, replica-plated to synthetic medium lacking uracil and the numbers of Ura− and Ura+ colonies determined after overnight incubation at 30°. *CAN1* mutation frequency was determined by dividing the number of Can+ Ura+ colonies by the number of viable cells plated for each spore colony. *CAN1* mutation rate was determined using the median *CAN1* mutation frequency from at least 10 independent trials (Lea and Coulson 1949). Statistical significance was tested by determining the number of trials with each strain that were above and below the group median frequency, and then performing $\chi^2$ analysis and Fisher’s exact test. This process was repeated at five successive growth intervals approximately 25 generations apart using single colonies that arose on the YPD viability plates.
Figure 2.  *CYH2* mutation rate does not increase in telomerase deficient cells during replicative senescence. *CYH2* mutation rate of ♦ wild type and ◇ est2Δ mutants was determined at the indicated time points. Wild type and est2 mutant spore colonies were obtained from freshly dissected tetrads of ABX1429 (Table 1) and dispersed in water. Aliquots were removed to determine viability following dilution, plating on to YPD medium and incubation for three days at 30°C. The remainder was plated on to synthetic medium containing 1 µg/ml cycloheximide and incubated for 3 days at 30°C. *CYH2* mutation frequency was determined by dividing the number of Cyh<sup>r</sup> colonies by the number of viable cells plated for each spore colony. *CYH2* mutation rate was determined using the median *CYH2* mutation frequency from at least 10 independent trials (Lea and Coulson 1949). Statistical significance was tested by determining the number of trials with each strain that were above and below the group median frequency, and then performing χ² analysis and Fisher’s exact test. This process was repeated at two additional growth intervals approximately 25 generations apart using single colonies that arose on the YPD viability plates.

Figure 3. Rev1, Rev7 and Rad30 do not significantly affect senescence and subsequent recovery.  a. ▲ wild type, ● rev7Δ, ▼ rev1Δ, ▼ rad30Δ, b. △ est2Δ, ○ est2Δ rev7Δ, □ est2Δ rev1Δ, ▽ est2Δ rad30Δ, × rad30Δ rev7Δ, and ◇ est2Δ rad30Δ rev7Δ. Serial liquid growth was performed as described previously (Hackett et al. 2001). During each day of serial liquid growth, hemocytometer counts were performed to determine the number of cell bodies, after which approximately 500 cells were plated to YPD and incubated at 30°C for 3 days. Colonies were then counted and divided by 500 to
determine plating efficiency. Finally, viability was determined following each day of
growth in liquid culture by taking the number of cell bodies, and multiplying by the
plating efficiency. Results are the mean ±2SE from at least eight independent samples of
each indicated genotype.
Figure 1

(a)  

(b)
Figure 2

`CYH2 Mutation Rate (Mutations/cell/generation)`

- **WT**
- **est2**

# Generations