New telomeres in yeast are initiated with a highly selected subset of TG₁₋₃ repeats

Kate M. Kramer and James E. Haber¹

Rosenstiel Basic Medical Sciences Research Center and Department of Biology, Brandeis University, Waltham, Massachusetts 02254-9110 USA

The creation of new telomeres was studied by generating a site-specific double-strand break in diploid strains of *Saccharomyces cerevisiae* that are unable to carry out homologous recombination. New telomere formation occurred ~1% of the time but only when (T₂G₄)₁₃ was present proximal to the break site. About half of the healing events occurred at a number of 1- to 9-bp G or G,T sequences located as far as 128 bp distal to the T₂G₄ repeats. Surprisingly, in 16 events at sites ending in GTGG, the first TG₁₋₃ nucleotides added always included either an 11- or a 13-bp sequence (GTGTGGGTGTG or GTGTGTGGGTGTG), after which each new telomere diverged into a less ordered TG₁₋₃ pattern. Moreover, at 75% of the remaining addition sites, these same 11- or 13-bp sequences were found overlapping the junction between the chromosomal primer and the newly added sequences. We propose that short G,T sequences near an organizing sequence such as (T₂G₄)₁₃ can act as primers to pair with the template RNA of a telomerase and add new sequences that are complementary to that RNA.

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The stability of linear chromosomes depends on the presence of telomeres at their ends. Telomeres function both to ensure the complete replication of chromosome ends and to protect them from degradation, recombination, and fusion with other chromosomes (for review, see Blackburn 1992; Blackburn and Szostak 1984; Zakian 1989; Biessmann and Mason 1992). Most eukaryotic telomeres consist of G-rich simple repeat sequences at the chromosome terminus, for example, T₂G₄, T₄G₄ or T₂AG₃. The conservation of telomere sequences among evolutionarily diverse organisms suggests that they all share a common structure and function. The mechanism by which telomeric repeats are added onto a preexisting array have been well documented in ciliates (Greider and Blackburn 1985, 1987, 1989; Zahler and Prescott 1988; Shippen-Lenz and Blackburn 1989) and in both mice and humans (Morin 1991; Prowse et al. 1993). In these cases, highly regular telomere repeats are added by an RNA-dependent DNA polymerase (telomerase) in which the 3' end of the preexisting telomere sequence acts as a primer for the addition of new telomere repeats. It is not yet clear, however, whether the yeast *Saccharomyces cerevisiae* telomere repeats, an irregular array of TG₁₋₃ sequences, are added by a telomerase mechanism.

In some circumstances, telomeres are synthesized de novo. New telomeres can be created as part of a geneti-

cally programmed phase of cellular development and differentiation. For example, the *Tetrahymena* telomerase adds telomere repeats to the ends of fragmented chromosomes during macronuclear development, at sites where no telomeric repeats are present (Yu and Blackburn 1991). Similar programmed telomere addition also occurs in *Ascaris* (Müller et al. 1991). New telomeres can also be formed to repair chromosomes broken by mechanical rupture or other stress. McClintock (1941) first described such "healing" of broken chromosomes, and similar events have been documented in yeast (McCusker and Haber 1981; Haber and Thorburn 1984; Surosky et al. 1986; Jäger and Philippsen 1989; Kämper et al. 1989). Spontaneously truncated chromosomes have also been found in organisms as diverse as malaria (Pologe and Ravetch 1988; Scherf and Mattei 1992) and humans (Wilkie et al. 1990).

In *S. cerevisiae* new TG₁₋₃ telomeric sequences are efficiently added onto transformed linear plasmids carrying heterologous telomere repeats from a variety of organisms (for review, see Zakian 1989; Biessmann and Mason 1992). There is yet no direct evidence of telomerase activity in yeast; however, a strong candidate gene for the yeast telomerase has been described in the gene *EST1* (Lundblad and Szostak 1989). *RAD52*-independent recombination has been proposed as an alternative mechanism for the addition and maintenance of yeast TG₁₋₃ (Wang and Zakian 1990b; Biessmann and Mason

¹Corresponding author.

1992). This suggestion is based on the observation of recombination between heterologous *Tetrahymena* T_2G_4 repeats and *Oxytricha* T_4G_4 repeats on opposite ends of a newly introduced linear yeast artificial chromosome (Pluta and Zakian 1989; Wang and Zakian 1990a). On the basis of these results it has been argued that yeast chromosome ends may sometimes acquire telomeric TG_{1-3} de novo by a *RAD52*-independent recombinational pathway. However, this model does not easily account for how new telomeres could be added on to nontelomeric sequences that frequently intervene between heterologous telomere repeats on transformed linear plasmids and the newly added TG_{1-3} (Murray et al. 1988; Wang and Zakian 1990b). It has been argued that this may occur by a copy choice mechanism or slippage during DNA synthesis (Wang and Zakian 1990b; Biessmann and Mason 1992).

To learn more about the way that new telomere addition occurs in yeast, we have developed a simple system to generate specific chromosome breaks that can be repaired by de novo telomere formation. Our goal was to examine the actual sequences added at new telomeres, to establish whether these events were likely to have arisen by telomerase activity or by recombination. Our sequence analysis of new telomere addition sites leads us to propose that new TG_{1-3} sequences are added by the action of a telomerase rather than by recombination. We base our model on the following observations. First, new telomere sequences can be added in a *RAD52* independent manner to short stretches (1–9 bp) of G, T sequence located up to 100 bp distal to an essential $(T_2G_4)_{13}$ organizing sequence. In some cases, the substrates for addition are as short as a single G, GT, GG, or GTG. Second, the junction at the chromosomal addition site frequently

contains one of two specific 11- or 13-bp motifs. Finally, the specific TG_{1-3} sequences that are added to a given site are strongly influenced by the DNA sequence at that site.

Results

Generating terminally deleted chromosomes

The strategy that we have used is illustrated in Figure 1. A site-specific chromosome break is introduced at the mating-type (*MAT*) locus on chromosome III by galactose-regulated induction of the *HO* endonuclease gene (Jensen and Herskowitz 1984; Connolly et al. 1988). In the absence of *RAD52* function, this double-strand break (DSB) cannot be repaired by homologous recombination, either with the silent copy donor sequences *HML* or *HMR* or with the *MAT* information on the opposite chromosome (Weiffenbach and Haber 1981). Diploids containing terminally deficient, healed chromosomes can then be recovered by genetic screening. Ten kilobases proximal to the break site, the broken chromosome carries the *URA3* marker and 13 T_2G_4 repeats flanked on the distal side by nontelomeric DNA. We reasoned that if new telomere formation could not occur at any of the sequences in the 10-kb interval adjacent to the break site, the T_2G_4 repeats would act as a possible "default" site.

Diploids of strain PA104T×RS200L (Table 1) carrying terminally deficient chromosomes are expected to be *Ura3*⁺ but *Thr4*[−]; therefore, strains with this phenotype were analyzed for their karyotype by contour-clamped homogeneous electric fields (CHEF) gel electrophoresis and Southern blot analysis (see Materials and methods).

Figure 1. Generating a terminally deficient chromosome III. Schematic diagrams of chromosome III are shown. (A) The *rad52* strain PA104T was marked by an insertion of *URA3::[TTGGGG]₁₃::pBR322*, 10 kb proximal to the *HO* cut site. [In strains YKK104BX1, YKK104BX2, and YKK104BX3 the pBR322 sequences are replaced by BX1, BX2, and BX3 sequences (see Materials and methods)]. A DSB was generated at *MATα* on chromosome III in PA104T following *HO* endonuclease expression from a *GAL::HO* plasmid. The DSB is degraded by extensive 5' → 3' exonuclease activity (White and Haber 1990) such that *MATα* gene functions are disrupted. The degradation of ~400 bp of α sequences causes cells to become phenotypically *Matα2*[−] *Matα1*[−] and exhibit an a-mating behavior (Weiffenbach and Haber 1981). These a-mating cells were then rescued by mating to either a *RAD52* (RS200L) or a *rad52* (YKK611) *MATα* haploid carrying the distal, recessive marker *thr4* on chromosome III. (B) The DSB was induced in a *MATα/MATα-inc* diploid (YKK910) homozygous for *rad52*. In the YKK910 diploid, only the *MATα* locus can be cut by *HO* endonuclease (Weiffenbach et al. 1983). Silent mating-type cassettes *HML* and *HMR* are indicated. (B) *Bam*HI; (S) *Sma*I; (H) *Hind*III; (X) *Xba*I; (R) *Eco*RI.

Table 1. Yeast strains

Strain	Genotype
PA104T ^a	<i>ho MATα rad52 trpΔ1 ura3-52 his4 ade2-101 [URA3::(T_2G_4)₁₃::pBR322] pFH800^b</i>
YKK104HO	<i>ho MATα rad52 trpΔ1 his4 ade2-101 [URA3::(T_2G_4)₁₃::pBR322] [lacZ::HOcs_{117bp}]^c pFH800</i>
YKK104ΔTG	<i>ho MATα rad52 trpΔ1 his4 ade2-101 [URA3::pBR322] pFH00</i>
YKK104BX1	<i>ho MATα rad52 trpΔ1 his4 ade2-101 [URA3::(T_2G_4)₁₃::BX1^d] pFH800</i>
YKK104BX2	<i>ho MATα rad52 trpΔ1 his4 ade2-101 [URA3::(T_2G_4)₁₃::BX2^d] pFH800</i>
YKK104BX3	<i>ho MATα rad52 trpΔ1 his4 ade2-101 [URA3::(T_2G_4)₁₃::BX3^d] pFH800</i>
RS200L	<i>ho MATα thr4 leu2 ura3-52 lys2-801 ade2-101</i>
YKK611	<i>ho MATα thr4 leu2 rad52::LEU2 ura3-52 lys2-801</i>
YKK901 ^e	<i>ho MATα-inc rad52 thr4 trp1H ura3-52 ade1</i>
YKK910	YKK901 mated to PA104T
YKK1010	YKK901 mated to YKK104HO
YKK910BX1	YKK901 mated to YKK104BX1
YKK910BX2	YKK901 mated to YKK104BX2
YKK910BX3	YKK901 mated to YKK104BX3

^aPA104T, YKK104HO, YKK104ΔTG, YKK104BX1, YKK104BX2, and YKK104BX3 are isogenic. [URA3::(T_2G_4)₁₃::pBR322] sequences are inserted 10 kb proximal to MAT (see Fig. 1).

^bpFH800, a TRP1 CEN4 plasmid carrying a GAL::HO gene was described by Nickoloff et al. (1989).

^cThe HOcs_{117bp} is described in Fishman-Lobell et al. (1992).

^dSee Fig. 3 for relevant sequences.

^eMATα-inc is described in Weiffenbach et al. (1983).

Southern blots of chromosome separating gels were probed with URA3 sequences (Fig. 2A) because the DSB was always generated on the copy of chromosome III carrying the inserted URA3 marker. In diploids with terminal deletions, the URA3-marked chromosome is dramatically shortened, consistent with its having lost sequences distal to the HO cut site. Diploids containing terminally deficient chromosomes were recovered in 0.2–1% of cells (Table 2), indicating that new telomere formation is not highly efficient.

Healing by de novo telomere addition requires T_2G_4 repeats

The chromosome in which the DSB was induced carried an inserted DNA fragment containing a URA3 marker, 13 repeats of *Tetrahymena* T_2G_4 and 341 bp of pBR322 DNA, all located 10 kb proximal to the HO cut site (Fig. 1). When we examined (by Southern blot analysis) DNA from diploids containing healed chromosomes, we found that all of them had undergone chromosomal rearrange-

Figure 2. Analysis of Ura⁺ Thr[−] diploids. (A) CHEF gel analysis of Ura⁺ Thr[−] diploids probed with URA3 sequences. The URA3 probe hybridizes to the *ura3-52* locus on chromosome V (top) and to URA3 inserted on chromosome III (top arrowhead). (Lane 1) Parental RAD52 strain (RS200L); (lane 2) parental *rad52* strain (PA104T) before the DSB is induced; (lane 3) a diploid containing a large internal deletion between MAT and its donor locus, HMR (Hawthorne 1963; Haber et al. 1980), was recovered and serves as a convenient marker (middle arrowhead); (lanes 4–10) Ura⁺ Thr[−] diploids carrying a terminally deleted chromosome III (bottom arrowhead). (B) Southern blot of genomic DNA from a terminally deleted chromosome III treated with exonuclease Bal31. A sample of genomic DNA was removed before addition of Bal31, and subsequent samples were removed at 10-min intervals. DNA was digested with HindIII before running on the gel. The probe is URA3. The

band corresponding to the URA3::(T_2G_4)₁₃ insert is indicated by an arrow; the other bands correspond to internally located URA3 sequences on chromosome V.

Table 2. Types and frequencies of DSB healing events

Strain background	Chromosome loss	Recombination	Terminal deletion
<i>rad52</i> (PA104T) × <i>Rad52</i> (YKK200L) ^a	229/701 (32%)	461/701 (66%)	7/1300 (0.5%)
<i>rad52</i> (PA104T) × <i>rad52</i> (YKK611) ^b	~2448/~2500 (97.9%)	22/~2500 (0.9%)	30/~2500 (1.2%)
<i>rad52/rad52</i> (YKK910)	~2267/~2300 (98.6%)	28/~2300 (1.2%)	5/~2300 (0.2%)
<i>rad52/rad52</i> (YKK1010) <i>lacZ::HOcs</i> _{117bp}	~4874/~5000 (97.5%)	20/~5000 (0.4%)	106/~5000 (2.0%)
<i>rad52</i> (PA104ΔTG) × <i>rad52</i> (YKK611) (T ₂ G ₄) ₁₃ deleted	~1280/1300 (98.5%)	20/~1300 (1.5%)	0/~1300 (<0.08%) ^c

Healed diploids were classified into three general categories by genetic phenotype and by CHEF gel analysis: (1) chromosome loss; (2) recombination; (3) terminal deletion. Numbers indicate independent healing events. For PA104T × YKK200L diploids a Ura⁻ Thr⁻ Leu⁻ phenotype was classified as chromosome loss, and a Ura⁻ Thr⁻ Leu⁺ phenotype was classified as healing by recombination; in all other strains, a Ura⁻ Thr⁻ phenotype was classified as chromosome loss, and a Ura⁺ Thr⁺ phenotype was classified as healing by recombination. In all strains a Ura⁺ Thr⁻ phenotype was classified as a terminal deletion. In PA104T × YKK200 and PA104ΔTG × YKK611 these Ura⁺, Thr⁻ diploids were confirmed by CHEF gel analysis.

^aData for terminal deletions in this cross were derived from a separate set of experiments from chromosome loss and recombination data.

^b(~)The number of total diploids was estimated from triplicate serial dilutions. Numbers for chromosome loss events were calculated as ~total diploids - the sum of recombination events + terminal deletion events.

^c*P* < 0.05, by a G-test (Sokal and Rohlf 1969).

ments very close to the inserted sequences. All terminally deleted chromosomes retained the *Sma*I site that marks the boundary between *URA3* and the T₂G₄ repeats, but none of them retained the *Hind*III site that is located ~350 bp distal to the T₂G₄ repeats (data not shown). This is significant because it means that there were no terminal deletion events at any other sites in the 10-kb interval between the site of the DSB and the *URA3*, T₂G₄, pBR322-containing insert.

We verified that the *URA3* marker was adjacent to the chromosome end in strains with a terminally deleted chromosome by treating genomic DNA in a time course with *Bal*31 exonuclease, which degrades sequences at the chromosome ends. Southern blots showed that the band corresponding to the *URA3* marker was sensitive to *Bal*31 exonuclease, indicating that *URA3* was next to the chromosome end (Fig. 2B).

Approximately half of the healing events in PA104T × RS200L involved addition of yeast TG₁₋₃ repeats distal to the *Tetrahymena* T₂G₄ segment. In ~41% (16/39) of the terminally deleted chromosomes, the *Bam*HI site marking the boundary between the T₂G₄ repeats and nontelomeric DNA was retained (data not shown), indicating that the new telomeres had been added to pBR322 sequences (Fig. 1). This result is consistent with previous studies reporting telomere addition to nontelomeric sequences immediately distal to T₂G₄ repeats on transformed DNA (Murray et al. 1988; Wang and Zakian 1990b). We observed a similar frequency of new telomere addition to nontelomeric sequences distal to the T₂G₄ repeats (41–24%) with three other isogenic strains described below, in which different sequences were substituted for pBR322.

The T₂G₄ repeats appear to be absolutely required for new telomere addition. When we repeated these experiments in isogenic diploid strains in which the *URA3* marker was inserted without accompanying T₂G₄ repeats (YKK104ΔTG × YKK611; Table 1), we observed no terminal deletions among ~1.3 × 10³ diploids (Table 2).

This result is statistically significantly different (*P* < 0.05) from the results obtained in the isogenic strain carrying the T₂G₄ repeats, based on a G-test (Sokal and Rohlf 1969).

Addition sites for yeast TG₁₋₃ are short stretches of G,T or G-rich DNA

We have analyzed in detail how new telomeres are formed by sequencing the sites of de novo addition of yeast TG₁₋₃ to broken chromosome ends. It is well documented that *Tetrahymena* T₂G₄ is a good substrate for the de novo addition of yeast TG₁₋₃ sequences (Szostak and Blackburn 1982; Shampay et al. 1984; Murray et al. 1988; Shampay and Blackburn 1989). We therefore chose to look at the cases where nontelomeric DNA was used as the addition site. Events such as these have been described before (Murray et al. 1988) but were not analyzed by DNA sequencing; hence, it was not clear whether particular sequences are required for such events or whether new yeast telomeres are added to random sequences near the T₂G₄ repeats. Forty-six independently isolated junctions containing new telomeres were cloned and sequenced (see Materials and methods). We found that in all 46 events that we have analyzed, very short stretches of G,T or G-rich telomere-like sequence were being used as substrates for yeast TG₁₋₃ addition (Fig. 3). Two of the 46 events (BX55 and BX3BC6) are unusual in that they contain deviations from the TG₁₋₃ pattern within the first 10 nucleotides of newly added telomere sequence; one has a run of 4 G's, and the other contains 2 consecutive T's, respectively. These exceptional events are not artifacts of cloning, as they were confirmed by analysis of independent clones. It is possible that they reflect errors of telomere addition caused by the very short stretches of G,T sequence to which they were added. We will confine further analysis to the 44 other healing events.

The sites of addition to be analyzed had pBR322 se-

Figure 3. Sequences of the sites of de novo telomere formation. pBR322 (BX), BX1, BX2, and BX3 refer to the original sequence to which new TG₁₋₃ repeats were added. BX1, BX2, and BX3 are synthetic *Bam*HI-*Xba*I fragments that were used to replace the *Bam*HI-*Hind*III pBR322 sequences of PA104T in strains YKK104BX1, YKK104BX2, and YKK104BX3, respectively (see Fig. 1). For BX3, sequences distal to the *Xba*I site are indicated in parentheses, as these sequences were used for de novo telomere addition in strain BX3CA2. Primer sequences used for TG₁₋₃ addition are in boldface type. Newly added TG₁₋₃ repeats are in uppercase letters. Consistently added 11-bp (ADD1) and 13-bp (ADD2) motifs are underlined. We have made the assumption throughout that sequences at the junctions that were present in the original strain are chromosomal sequences (for discussion, see text). (A) Ter

the parsimonious assumption throughout that any nucleotides that were in the original sequences were preserved, although it is certainly possible that part of the GGGTGTGGT region was degraded and resynthesized during telomere formation). In these three cases, the sequences that are added are all normal TG₁₋₃ sequences but not identical to each other. (2) One event apparently

involved “chewing back” most of the segment to add at the GG at the 5′ end. (3) Five events have added new telomere DNA after removing the terminal T. What is striking about these five independent events is that the first 11 nucleotides that were added are identical (GTGTGGGTGTG, underlined in Fig. 3A), after which the sequences diverge from each other to the unpredictable TG₁₋₃ pattern seen with the other new telomeres. This is a striking result suggesting that some sequences may act as primers for new telomere addition by “setting the frame” of the template so that the next sequences that are added are always the same. For further discussion, we will refer to this conserved, added GTGTGGGTGTG sequence as ADD1.

We also note that there does not appear to be any difference in the frequency of recovery (Table 2) or in the types of new telomere sequences that were added in the seven cases recovered from *rad52/rad52* diploids (telomeres BX611C, BX611D, BX611E, and BX611H and BX1025, BX1026, and BX1034 in Fig. 3A) and the remaining six cases obtained from *RAD52/rad52* cells. Consistent with this, de novo addition of TG₁₋₃ to the ends of transformed linear plasmids has been shown to be independent of *RAD52* function (Dunn et al. 1984; Zakian et al. 1985).

Particular primers promote addition of identical TG₁₋₃ sequences

To explore the effect of the primer sequence on the addition of yeast telomere sequences, we constructed three additional strains in which the pBR322 sequences containing the GGGTGTGGT motif were replaced by other possible substrates for telomere addition. The sequences distal to the *Bam*HI site, labeled BX1, BX2, and BX3, are presented in Figure 3. These modified regions were inserted into strains YKK104BX1, YKK104BX2, and YKK104BX3 as described in Materials and methods. Terminally deleted chromosomes were generated and analyzed as described above.

Construct BX1 differs from the original segment (BX) in three important respects (Fig. 3B). First, the circularly permuted sequence GGTGGGTGT was inserted in place of the preferentially used GGGTGTGGT sequence discussed above. Moreover, to see whether the original preferred site would be used preferentially when it was farther away, this motif was inserted ~90 bp away from the T₂G₄ sequences. Finally, we also included two repeats of the human telomere sequence TTAGGG as a possible addition site. Among 13 healing events analyzed, three used GGGTGTGGT at its more distant location. One (BX1-40) retained the distal T and proved to add an identical sequence to one of the previous examples (BX611E; Fig. 3A) for the first 12 bp. The two other new telomeres were apparently added after the removal of the distal T. Strikingly, in both cases, the same 11-bp ADD1 sequence was added as in the BX construct. Again, following ADD1, the two sequences diverged from each other and from the previous examples. This result substantiates the conclusion that there is a highly preferential addition

of the first 11 bp of new telomere sequence when the primer sequence is GGGTGTGG.

When we look at the other 10 healing events with construct BX1, 4 have added new sequences to the 3′ T of the circularly permuted site, but there is no distinctive order to the first 11 nucleotides added. Thus, not all G,T-rich regions appear to fix the order of initial new telomere addition. There were also two additions at the ends of either the first or second TTAGGG repeat; both included same first 20 nucleotides before the sequences diverged.

A different primer yields a different conserved, initially added telomere sequence

In the construct BX2, the original preferred site was replaced by TGGTGGTGG. This TG₁₋₃ sequence is never found within authentic telomeres, but it was readily used as a site for new telomere addition (Fig. 3C). Surprisingly, of eight new telomeres added to the end of this segment, three had initiated with the same GTGTGGGTGTG (ADD1) sequence. The remaining five events at this site had a perfectly conserved 13-bp sequence before the TG₁₋₃ sequences diverged from each other. This sequence, GTGTGTGGGTGTG, which we will term ADD2, is identical to ADD1 except for the presence of an additional GT at the 5′ end. Additionally, in the healing event in BX2CC3, the TGGTGGTGG region apparently had been “trimmed back” to TGGTGG before addition of the same ADD1 sequence. We note that the terminal 4 nucleotides, all of the ADD1 and ADD2 addition sites (Fig. 3), are GTGG.

Finally, with construct BX3 (Fig. 3D), the preferential site of addition was replaced by another circularly permuted version of the sequence in pBR322, TGTGGTGGG. Of nine events sequenced, there was no fixed order to the first 11 nucleotides added, and 4 new telomeres actually began at a single G separated from the 9-bp G,T sequence by a C. These data provide strong evidence that some primer sequences, but not others, promote the addition of highly ordered initial telomere sequence.

Effect of the position of the DSB on the efficiency of de novo telomere formation

Healing of a broken chromosome by de novo telomere formation is a rare event (0.2–1.0%) when it occurs 10 kb from the break site. In our experiments, the DNA between the HO cut site and the T₂G₄ repeats presumably must be degraded before the addition of yeast TG₁₋₃ can occur. Previous studies in our laboratory have shown that in *rad52* strains, an HO endonuclease cut is followed by extensive 5′ → 3′ exonuclease activity that can generate single-stranded 3′-ended strands as long as 10 kb (White and Haber 1990). Nevertheless, it was possible that the efficiency of new telomere formation could be improved by creating the DSB closer to the site of healing. We therefore repeated our experiments in an isogenic strain (YKK1010; Table 1) in which an HO cut site

was placed just 1.6 kb distal to the T_2G_4 repeats. We found that the frequency of healing by de novo telomere formation increased 10-fold from 0.2%–2% (Table 2). Thus, the efficiency of de novo telomere formation increases with decreasing amounts of intervening DNA between the break site and the site of new telomere addition.

Discussion

Analysis of telomeric DNA sequences added de novo during the healing of broken yeast chromosomes leads us to propose that new TG_{1-3} repeats are added by the action of a telomerase rather than by recombination based on the following observations: First, new telomeres can be added in a *RAD52*-independent manner at telomere-like G,T sequences that can be as short as a single G, GG, or GTG. Moreover, this addition is completely dependent on a nearby $(T_2G_4)_{13}$ organizing sequence. There are many equivalent short G,T stretches in the 10 kb between the HO cut site and the T_2G_4 repeats, yet none of them are used as telomere addition sites. Moreover, although recombination between poorly matched DNA sequences can occur at very short regions of identical sequence [Mézard et al. 1992], those events require a more extensive homologous alignment of sequences and are *RAD52* dependent. Second, the fact that ADD1 and ADD2 are invariably added to the end of some primers (GGGTGTGG or TGGTGGTGG) but not to others (i.e., GGTGGGTGT or TGTGGTGGG) is consistent with the constraints imposed by a telomerase during the first steps of creating a new telomere but is difficult to reconcile with a recombinational model. This is especially evident when the sequence GGGTGTGG always specifies ADD1, but the same sequence with an additional T at the end does not. A further argument for the creation of new telomeres by telomerase comes from an analysis of the frequency with which the ADD1 sequence is found directly after GGGTGTGG in nonjunctional telomere sequences. The sequence GGGTGTGG occurs 182 times in the more distal parts of the sequences analyzed (Fig. 3 and additional sequences not shown). In only 25 of these cases was GGGTGTGG followed by ADD1. In three other cases, the sequence was followed by ADD2. Thus, if new telomeres forming at GGGTGTGG were created by recombination, we would not have expected to find ADD1 at that site more than ~14% of the time.

A model for the creation of new telomeres adjacent to broken chromosome ends

We propose that broken chromosomes are healed by the action of a yeast telomerase, as shown in Figure 4. We imagine that in the initial step the action of a 5' → 3' exonuclease can degrade DNA from the site of the DSB until an appropriate primer region, in close proximity to the essential organizing sequence, is exposed. On the basis of the known mechanism of telomerase in *Tetrahymena* [Greider and Blackburn 1985, 1987; Blackburn

Figure 4. A model for the creation of a new telomere by telomerase. The telomerase RNA is shown associated with its protein component (large rectangle). Telomere binding proteins are represented as small circles. Telomerase recognizes the T_2G_4 repeats and/or these proteins. The end of the broken chromosome is degraded first by a 5' → 3' exonuclease. And endo- or exonuclease may then remove the 3' tail, thus exposing the primer. Once the DNA primer is exposed, the 3' end of the RNA template anneals to the end of the primer. (A) The primer ends in GTGG, which can specify the addition of ADD1. The DNA primer is extended by the addition of ADD1. (B) A primer not ending in GTGG aligns with the telomerase template in a different way and is extended by the telomerase such that ADD1 (underlined) is present overlapping the junction sequence.

1992) and the invariant addition of ADD1 and ADD2 to certain primers, we propose that the template RNA must include sequences complementary to ADD1 and/or ADD2. In the model presented (Fig. 4), the pairing site of the telomerase RNA template is a 3'-CACC-5' because, as discussed above, primers with GTGG at their terminus appear to align the template more precisely, resulting in the systematic addition of the same new sequence. Apparently, other priming sites do not have bases that are precisely complementary to the primer binding site of the telomerase template and thus do specify the addition of the entire template sequence.

However, at other sites of addition, we postulate that the telomerase primer pairs to the internal part of the 11- or 13-bp template (Fig. 4B). A strong prediction of this model is that ADD1 or ADD2 should overlap the junc-

tion between the primer and newly added sequences. An examination of all junction sequences, described below (Fig. 5), confirms this prediction; in 37 of 44 examples, the junction between the chromosomal healing site and the new sequences contains either ADD1 or ADD2. In addition to the 16 instances in which ADD1 or ADD2 are appended directly to the chromosomal primer sequence GTGG (Fig. 5A), 21 of the 28 remaining healing events have either an ADD1 or ADD2 sequence overlapping the junction between the original and the newly added sequences (Fig. 5B). We note that in three cases listed at the top of this set, the same primer has been used to create both ADD1 and ADD2 junctions.

The last set (Fig. 5C) comprises five cases in which the new telomere sequences begin with one or more GT re-

peats followed by GG rather than the GGG that is found in the 37 examples presented above. In these events we find that the junction of original and new sequences contains the sequence GTGTGGTGTG, which is a 10-bp variant of ADD1, lacking one of the three central G's. This may represent another consensus sequence, ADD3. We note that in the more distal parts of the TG₁₋₃ sequences we have analyzed, about 1/3 (150/433) have (GT)_nG₂ and the rest have (GT)_nG₃. Yet only 6 of 44 of the initial telomere sequences have GG. This is statistically significantly different ($P < 0.05$) from the overall frequency of GG versus GGG and again emphasizes that the initial addition of telomere sequences is more precisely dictated than random TG₁₋₃ telomere sequences.

The two remaining events (Fig. 5D) do not fit easily into any category and illustrate the difficulty in generating a model that accounts for the unusual variability of the yeast telomere repeat. This difficulty is also evident in considering how sequences are added after the establishment of new telomeres with ADD1 or ADD2. For example, an inspection of the seven events in which ADD1 was added to GGGTGTGG in strains BX and BX1 fails to reveal a clear pattern of subsequent TG₁₋₃ sequence addition. One cannot account for this variability by assuming that telomerase simply resynthesizes the ADD1 or ADD2 (or even ADD3) consensus with each new round of TG₁₋₃ addition. This suggests that the mechanisms involved in the creation of a new telomere must be different, in some respects, from those involved in maintaining a preexisting telomere. One obvious difference is that existing telomeres contain many protein-binding sites such as RAP1 sites (Longtine et al. 1989; Liu and Tye 1991), which appear to play an important role in telomere maintenance (Longtine et al. 1989; Lustig et al. 1990; Sussel and Shore 1991; Kyrion et al. 1992). Preexisting telomeres may also form important tertiary structures, such as G-quartets (Williamson et al. 1989; Kang et al. 1992), which may play a role in telomere maintenance.

We have observed that in some cases, the same primer can be used to create both ADD1 and ADD2 junctions (Fig. 5). There might be a family of telomerase RNAs with each template specifying a different sequence, so that the use of different templates would give rise to the irregular TG₁₋₃. It has been suggested, on the basis of telomere sequence analysis, that multiple templates might exist in *Paramecium* and *Plasmodium* (Blackburn 1992). Alternatively, it is possible that one template can be aligned in two equally likely ways. In vitro studies of the human telomerase (Morin 1991) support the idea that flexibility in primer alignment might cause variability in patterns of addition, which in the case of yeast might be the characteristically variable TG₁₋₃. In addition to these models, it has been suggested that TG₁₋₃ irregularity could be caused by slippage on the RNA template (Shippen-Lenz and Blackburn 1989).

Although our results are best explained by the action of a telomerase, under special circumstances recombination between related, but not identical G,T repeats may play an important role in telomere formation. The direct

5' adjacent sequence	sequences at the addition site	number of events
A.		
gggtgtgg	GTGTGGGTGTG	7
tggtgtgg	GTGTGGGTGTG	3
tggtgg	GTGTGGGTGTG	1
tggtgtgg	GTGTGGGTGTG	5
		16
B.		
gggtgtg	gtGTGGGTGTG	1
gggtgtg	gtGTGGGTGTG	2
ggtgg	gtgtGGGTGTG	2
ggtgg	gtgtGTGGGTGTG	1
tgtggtggc	gTGTGGGTGTG	1
tgtggtggc	gTGTGGGTGTG	1
tgtggtgg	gTGTGGGTGTG	2
tgtggt	gTGTGGGTGTG	1
ttagggttagg	gTGTGGGTGTG	1
ttagg	gTGTGGGTGTG	1
ggtgggtg	gTGTGGGTGTG	1
g	gtgTGGGTGTG	1
gg	gtgTGGGTGTG	1
gt	gTGTGGGTGTG	1
acg	gTGTGGGTGTG	1
tg	gTGTGGGTGTG	1
gta	gtGTGGGTGTG	1
aga	gtGTGGGTGTG	1
		21
C.		
gggtgtg	gtGTGGGTGTG	1
ggtggg	gtgtGGGTGTG	1
tgg	GTGTGGGTGTG	1
gg	gtGTGGGTGTG	1
tgtggtgg	gTGTGGGTGTG	1
		5
D.		
cta	gTGTGTGGGTGTG	1
tgtggtggc	gTGGTGTG	1
		2

Figure 5. Patterns of de novo TG₁₋₃ addition. Chromosomal primer sequences for de novo telomere addition are highlighted, and newly added sequences are in uppercase letters as in Fig. 3. (A) Events in which complete ADD1 or ADD2 is added to chromosomal priming sites with GTGG at the 3' end; (B) events in which the ADD1 or ADD2 motif is present at the junction overlapping the primer and the newly added sequences; (C) events in which GG rather than GGG is found in the first G cluster of newly added ADD3 telomere sequence GTGTGGTGTG.

joining between T_4G_4 and T_2G_4 sequences demonstrated by Wang and Zakian (1990b) argues strongly in favor of recombinational joining in this case. Quite likely both types of processes are possible, especially in the stabilization of transformed DNA.

A role for an organizer sequence such as $(T_2G_4)_n$

On the basis of our data and that of others [Murray et al. 1988; Shampay and Blackburn 1989], we propose that telomerase in yeast is incapable, itself, of establishing a new telomere. It requires the presence of an organizer sequence such as $(T_2G_4)_n$ nearby. This is similar to the results of in vitro studies of *Tetrahymena* telomerase [Harrington and Greider 1991], where new telomere sequences can be added at a distance from the T_2G_4 repeats. In contrast, an organizing sequence does not appear to be needed to add new telomeres at chromosomal truncations in malaria [Scherf and Mattei 1992] and humans [Wilkie et al. 1990; Morin 1991] and, in at least one developmental stage, *Tetrahymena* [Yu and Blackburn 1991].

We imagine that certain proteins can bind to the organizer region [several possible candidate proteins have been identified [Liu and Tye 1991; Brigati et al. 1993]] and stabilize the interaction of yeast telomerase with short G,T stretches upstream, allowing them to be used as an initial primer of a new telomere. In some cases, additional proteins that bind to the primer itself may play an important role.

We have attributed the preferential addition of ADD1 and ADD2 at the beginnings of new telomeres to a preferred interaction with telomerase template RNA(s). It is also possible that the preferential appearance of ADD1 and ADD2 at these junctions does not come from preferential addition but from a preferential retention of these sequences. Newly formed telomeres may be degraded by exonucleases unless they have sufficient tertiary structure or are protected by associating with telomere-binding proteins such as RAP1 [Lustig et al. 1990]. In this view, only those additions of new sequences that established protein binding would be retained. The absence of preexisting telomere-binding proteins bound at the broken chromosome end may account for the imprecision of telomere addition (GGGG or TT) in the two cases noted above. In more distal regions of new telomeres we also found the presence of an A residue in BX39 and a TT in BX65 (Fig. 3A). These could also be errors of telomere addition but might also be attributable to PCR artifacts because only a single subclone from each of these strains was sequenced.

The system that we have set up will allow us to explore a number of other questions about the formation of new telomeres. For example, how is the DNA removed between the DSB and the site of new telomere addition? Our physical monitoring studies of homologous recombination induced by DSB [White and Haber 1990; Ray et al. 1991; Fishman-Lobell et al. 1992; Sugawara and Haber 1992] have all indicated that there is extensive $5' \rightarrow 3'$ exonuclease digestion of HO-cut DNA but little

$3' \rightarrow 5'$ exonuclease activity. How, then, is the long $3'$ -ended strand replaced by a telomere? In homologous recombination events, such nonhomologous DNA must be removed by an apparently endonucleolytic process that requires the UV repair RAD1 gene [Fishman-Lobell and Haber 1992]. Experiments are under way to determine whether new telomere formation also requires this activity.

We are also interested in exploring the way in which the T_2G_4 region acts at a distance in the formation of new telomeres and what processes limit the efficiency of new telomere formation. If we can increase the efficiency of new telomere formation further, we should be able to follow these events by the same physical monitoring events used to describe HO-mediated recombination.

Materials and methods

Strains

Parental yeast strains are listed in Table 1. Strain PA104T was marked by an insertion of $URA3::(TTGGGG)_{13}::pBR322$, 10 kb proximal to the HO cut site in *MATa*. The insertion was constructed in plasmid pJH154 that contains a 3.5-kb *EcoRI*–*Bam*HI chromosome III fragment in pBR322. This fragment was originally provided in phage λ Charon 4A clone CP2 by John Larkin and John Woolford (Carnegie Mellon University, Pittsburgh, PA). The 1.1-kb *URA3* gene $(TTGGGG)_{13}$ and the 0.27-kb *Bam*HI–*Hind*III fragment of pBR322 were inserted into the chromosome III segment at a unique *Hind*III site (see Fig. 1). This construct was then transplanted [Rothstein 1983] into chromosome III as an *EcoRI*–*Sal*I fragment, with the nonhomologous *Sal*I end being lost during recombination.

Strains YKK104BX1, YKK104BX2, and YKK104BX3 were constructed by replacing the pBR322 sequences in PA104T with *Bam*HI–*Xba*I fragments BX1, BX2, and BX3 (Fig. 3). These sequence transplacements were accomplished by transformation of DNA fragments carrying BX1, BX2, and BX3 sequences flanked by chromosome III sequences into PA104T. Strain YKK104HO was constructed by integrating a 2.6-kb *Pvu*II fragment of the *lacZ* gene carrying an inserted 117-bp HO cut site [Fishman-Lobell et al. 1992] just distal to the pBR322 sequences in plasmid pJH154 so that the insert transplanted into chromosome III of PA104T carried an HO cut site 1.6 kb from the T_2G_4 repeats. Strain YKK104 Δ TTG was constructed by replacing $URA3::(TTGGGG)_{13}::pBR322$ with $URA3::pBR322$. All of these constructs involved transplacements into PA104T, a *rad52* strain and, thus, were done after transformation with a *CEN4 ARS1* plasmid carrying a *HIS4* marker and a wild-type *RAD52* gene (pKK5) to allow integration of transformed DNA. After integration of the relevant DNA fragment, pKK5 was lost by growth on YPD. All yeast transformations were done according to Schiestl and Gietz 1989).

Bacterial strains used were DB6507 ($r^- m^- pro^- gal^- recA^- leuB^- thr^- str^- pyrF::Tn5$) for selection of plasmids carrying *URA3* and *Escherichia coli* SURE (*uvrC umuC sbcC recJ recB hsdR mcrTA mcrB endA mrr*) and MC1061 (*hsdR mcrB araD139 Δ (araABC-leu)7679 Δ lacX74 galU galK rpsL thi*) for selection of *amp^r* plasmids.

Creation of DSBs

Rich medium (YPD), nonfermentable medium (YP-lactate), and selective media (SD) were as described [Sherman et al. 1983;

Rudin and Haber 1988). Terminally deleted chromosomes were isolated using two general strategies (see Fig. 1). 1) The DSB was generated in haploid PA104T, which was then mated to RS200L (Rad⁺) or YKK611 (Rad⁻), and Ura⁺ Thr⁻ derivatives were selected for analysis. To generate the DSB, haploid strain PA104T was grown in YP-lactate medium, and *GAL::HO* gene on plasmid pFH800 (Nickoloff et al. 1989) was induced with 2% galactose for 1 hr. Cells were washed in YPD, pelleted, and mixed as a thick suspension with an approximately equal number of RS200L or YKK611 cells and allowed to mate overnight. Serial dilutions were plated on SD-Trp and grown for 2 days. Ura⁺ Thr⁻ colonies were purified and subsequently analyzed. To ensure that Ura⁺ Thr⁻ derivatives came from independent events, only one isolate from each mating was retained. (2) The DSB was generated in diploid strains YKK910, YKK1010, YKK910BX1, YKK910BX2, or YKK910BX3 (Table 1) as described above. Ura⁺ Thr⁻ derivatives were selected for analysis. Serial dilutions were plated onto SD-Trp and allowed to grow for 2 days. Colonies were transferred to SD-Ura and SD-Thr and Ura⁺ Thr⁻ colonies were purified and subsequently analyzed. All incubations were at 30°C.

CHEF gel analysis

Chromosomal DNA was separated on agarose gels as described (Carle and Olson 1985; Chu et al. 1986; Louis and Haber 1992). The chromosomal DNA was transferred to a Bio-Rad Zeta-probe nylon filter as per the manufacturer's instructions. Filters were hybridized with *URA3* sequence probe [α -³²P] CTP labeled by random primer extension (Feinberg and Vogelstein 1984; Southern 1975).

BAL 31 digestion

Fifty micrograms of genomic DNA was digested with 0.5 units of Bal31 nuclease (NEB) in a total volume of 500 μ l of 600 mM NaCl, 12 mM CaCl₂, 12 mM Tris HCl at pH 8.0, 1 mM EDTA (30°C). A 5- μ g sample was removed before addition of the enzyme and at 10-min intervals after enzyme addition. Ten microliters of 0.25 M EGTA was added to stop the reaction, and the DNA was precipitated with 2 volumes of ethanol. The DNA was pelleted, resuspended in 20 μ l of TE buffer, and digested with HindIII (NEB). The DNA was run on an 0.8% agarose gel, and the DNA was transferred to a Bio-Rad Zeta-probe nylon filter as per the manufacturer's instructions. Filters were hybridized with [α -³²P] CTP-labeled *URA3* sequence probe as described above.

Cloning and sequencing of de novo telomeres

The critical step in obtaining these sequences was to amplify the relevant DNA using PCR (Saiki et al. 1988). Because all of the more distal sequences were the highly repetitive yeast TG₁₋₃ sequences, it was necessary to treat genomic DNA with BAL 31 exonuclease or T4 DNA polymerase to eliminate the 3' overhang at the end of the chromosome (Henderson and Blackburn 1989; Wang and Zakian 1990a) and then ligate the telomere end to a unique template for PCR amplification. The amplified DNA was then subcloned into a sequencing vector to obtain sequences of the junction. In one case, the relevant sequences were cloned by ligation of the cloning vector pUC9 (Vieira and Messing 1982) directly onto the end of the chromosome after Bal31 treatment (Van der Ploeg et al. 1984).

Genomic DNA was treated in one of two ways.

Ligation to a double-stranded synthetic oligonucleotide A double-stranded oligonucleotide was made by allowing two complementary oligonucleotides to anneal

oligo 1, 5'-CATTGCTGCTGCCGGTCATTCGAACCC-3'
oligo 2, 3'-GTAAACGACGACGGCCAGTAAGCTTGGG-5'

When annealed, these yield a 26-bp HindIII linker (HindIII site underlined). Approximately 5 μ g of genomic DNA was treated with 3 units of T4 DNA polymerase for 20 min at 16°C in a total volume of 50 μ l in 1 $\times$ New England Biolabs (NEB) buffer 4, to create blunt DNA ends. T4 DNA polymerase was inactivated by incubation at 75°C for 10 min. Three hundred nanograms of T4-treated genomic DNA was ligated to 20 ng of double-stranded oligonucleotide using NEB T4 DNA ligase in the buffer provided in a total volume of 50 μ l for 4 hr or overnight at 16°C. The EtOH-precipitated DNA was digested with HindIII and separated on a 0.5% agarose gel. HindIII fragments of 1.2–1.9 kb were cut out of the gel and purified using Prep-a-gene (Bio-Rad).

Circularization and amplification of a URA3–telomere region Approximately 5 μ g of genomic DNA was digested overnight with HindIII in a total volume of 400 μ l. Digested DNA was treated with 1 mg/ml of RNase A for 30 min. The digestion mixture was treated with 1:1 vol/vol phenol/chloroform and precipitated in EtOH in the presence of 0.3 M sodium acetate. The genomic DNA was then treated with T4 DNA polymerase as described above and then heat inactivated. The DNA was diluted to ~2–3 μ g/ml and incubated in the presence of NEB T4 DNA ligase in the buffer provided at 16°C for 4 hr or overnight to promote intramolecular ligation. The DNA was then precipitated with EtOH in the presence of 0.3 M sodium acetate and resuspended in TE buffer.

Following self-ligation or ligation to a double-stranded synthetic oligonucleotide, the DNA was amplified by PCR (Saiki et al. 1988) using a Techne Programmable Dri-Block PHC-1 temperature cycling machine. The oligonucleotides used were synthesized with a Cyclone V1.05 synthesizer using phosphoramidite chemistry. Primers used for DNA amplifications were oligo 2 shown above, and

oligo 3, 5'-TTGGGGTCTGCCTCGCG-3'

oligo 4, 5'-TCTGTGCTCCTTCCTTCGTTCTTC-3', a *URA3* primer

The enzymes used for DNA amplification were either PyroTase (Molecular Genetic Resources, Inc.) or Deep Vent Polymerase (NEB).

Primers were removed from the PCR product by treating with Prep-a-gene (Bio-Rad). The amplified DNA was subcloned into a pGem3Z vector (Promega). Sequencing was done directly from plasmid miniprep DNA isolated by the boiling method (Maniatis et al. 1983). Using the fmol sequencing kit, (Promega) ~50 ng of miniprep DNA was used in each sequencing reaction. In some cases, two or more subclones from the amplified DNA were sequenced (see Fig. 3). In every case where multiple subclones were sequenced, the initially added TG₁₋₃ repeats were the same.

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K M Kramer and J E Haber

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