 Nucleic Acids Research, 1998, Vol. 26, No. 23 **5365–5371**

Processing of telomeric DNA ends requires the passage of a replication fork

Isabelle Dionne and Raymund J. Wellinger*

Département de Microbiologie et Infectiologie, Faculté de Médecine, Université de Sherbrooke, 3001 12ème Avenue Nord, Sherbrooke, Québec J1H 5N4, Canada

Received August 11, 1998; Revised and Accepted October 19, 1998

ABSTRACT

During telomere replication in yeast, chromosome ends acquire a long single-stranded extension of the strand making the 3′ end. Previous work showed that these 3′ tails are generated late in S-phase, when conventional replication is virtually complete. In addition, the extensions were also observed in cells that lacked telomerase. Therefore, a model was proposed that predicted an activity that recessed the 5′ ends at yeast telomeres after conventional replication was complete. Here, we demonstrate that this processing activity is dependent on the passage of a replication fork through yeast telomeres. A non-replicating linear plasmid with telomeres at each end does not acquire single-stranded extensions, while an identical construct containing an origin of replication does. Thus, the processing activity could be associated with the enzymes at the replication fork itself, or the passage of the fork through the telomeric sequences allows a transient access for the activity to the telomeres. We therefore propose that there is a mechanistic link between the conventional replication machinery and telomere maintenance.

INTRODUCTION

Telomeric DNA of most eukaryotic organisms is made of short tandem repeats (reviewed in 1,2). Due to the nature of the repeats, one strand usually is rich in guanines and the complementary strand is rich in cytosines, and they are commonly abbreviated as the G- and C-strand, respectively. In all organisms tested, the G-strand runs 5′ to 3′ toward the end of the chromosomes and forms a single-stranded overhang at the physical termini (3–6). Telomeres are essential for chromosome stability and cell viability as they protect chromosome ends from random fusion events and degradation and they ensure the complete replication of the chromosome (1,7,8).

All conventional DNA polymerases require a primer with a free 3′-OH group as well as a template, and they synthesize DNA in the 5′ to 3′ direction. Given these properties, conventional replication is expected to leave short primer-sized gaps on the 5′ end of the strands that were generated by lagging-strand synthesis (1,7,8). Upon successive divisions, the telomeres will slowly but progressively lose sequences from the end until eventually all telomere functions are lost, chromosomes become unstable and, without appropriate rescue events, the cells die (9,10). However, a telomere-specific reverse transcriptase, called telomerase, can elongate short single-stranded G-strand in a DNA template independent manner and is crucial for a stable maintenance of telomeric repeats (8,10,11).

In yeast, the repeats at the chromosomal termini can be abbreviated as $TG_{2–3}(TG)_{1–6}$, or simply $TG_{1–3}$, and there are ~250–400 bp of these repeats at each telomere (1,8,12). An analysis of the replication intermediates formed at yeast telomeres demonstrated the occurrence of single-stranded extensions of the G-strand (G-tails) of ≥30 bases late in S-phase, after the replication fork reaches the telomeres (13,14). Such G-tails can be generated on both ends of a linear plasmid (15). Furthermore, even in the absence of *TLC1*, the RNA component of telomerase, G-tails still form in the same cell-cycle-regulated manner as observed for wild-type cells (15,16). These observations led to the proposal of a 5′–3′ exonuclease or other activity that recesses the 5′ end of the $C_{1–3}A$ strands to generate 3′ overhangs at both ends of each chromosome as a late step in chromosome replication (15,16). Those G-tails could then be a substrate for telomerase and, concomitant to, or after telomerase mediated elongation, the conventional DNA replication machinery could fill in the overhangs, leaving short 3′ overhangs at both ends of each chromosome (7,15). Proteins that bind specifically to a short overhang of the G-rich strand have been identified in *Oxytricha* and related ciliates (17–19), and recently it was shown that homologs of the mammalian Ku-proteins function as terminus binding factors in yeast (20, reviewed in 21).

Since G-tails can be generated in cells that are devoid of telomerase, the question arises as to whether the activity required to produce them is dependent on a passage of the regular replication machinery or whether they are generated by a cell-cycle-regulated process that is independent of the actual replication of telomeres. To address this question, we used two linear plasmids which, after an endonucleolytic cleavage *in vivo*, differed in that one contained an origin of replication and the other one did not (Fig. 1). If the exonuclease is dependent on replication fork passage, G-tails will only be detected for the plasmid containing an origin of replication, but not for the one that was non-replicating. Using non-denaturing Southern hybridization and two-dimensional (2D) agarose electrophoresis, we demonstrate

*To whom correspondence should be addressed. Tel: +1 819 564 5214; Fax: +1 819 564 5392; Email: r.wellin@courrier.usherb.ca

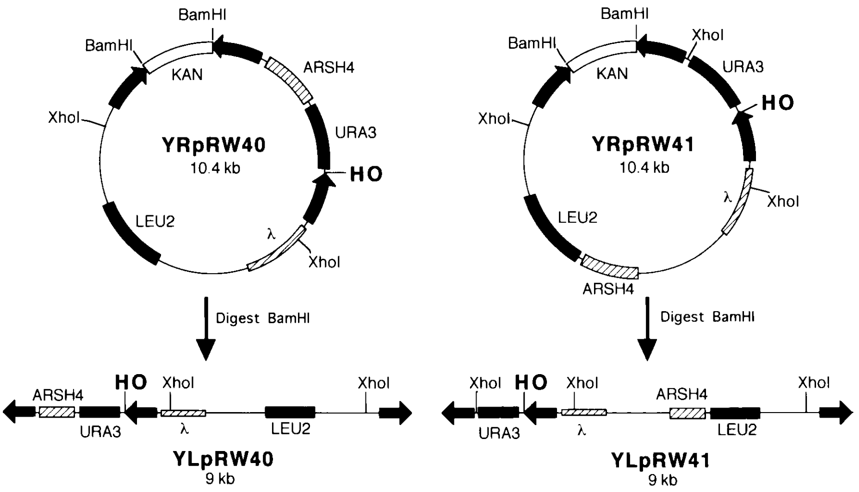

Figure 1. Structure of the plasmids used. Only relevant restriction sites are indicated. The bold arrows denote the 280 bp tracts of telomeric $C_{1-3}A/TG_{1-3}$ sequences. Digestion of the circular plasmids YRpRW40 and YRpRW41 with *Bam*HI yields linear fragments YLpRW40 and YLpRW41 that were used to establish the plasmids in the yeast cells. After *HO* endonuclease-mediated cleavage of YLpRW40 and YLpRW41, the plasmids are shortened to 7 and 7.4 kb linear plasmids, respectively. Note that the final linear plasmids contain one natural telomere established *in vivo* (right end on the drawing) and one telomere with ~50 bp non-telomeric sequences at the ends (left end, after *HO*-cut).

here that G-tail formation on plasmid ends only occurs if the plasmid is replicating. These results imply that the activity creating G-tails is either directly associated with the replication fork machinery or is dependent on fork passage to allow its access to chromosomal termini. Thus, the data support a model in which the passage of a replication fork is required to form G-tails at the telomeres at the end of S-phase. Since these overhangs are the required substrate for telomerase-mediated telomere maintenance, the data suggest a direct mechanistic link between conventional replication and telomerase.

MATERIALS AND METHODS

Plasmids and strains

Plasmids YRpRW40 and YRpRW41 were produced as follows: the yeast shuttle vector pRS305 (22) containing the *LEU2* gene and in which the *Nae*I site was changed to an *Xho*I site served as base vector. Inserted into this vector were a 1.45 kb *Sal*I–*Nhe*I fragment derived from λ DNA (nt 33244–34679), a 300 bp *Eco*RI–*Kpn*I fragment containing 280 bp of $C_{1-3}A/TG_{1-3}$ DNA and isolated from pYLPV (14), an *HO*-cut site on a 139 bp *Hin*dIII–*Eco*RI fragment (23,24), a 1.1 kb *Hin*dIII–*Hin*dIII fragment containing the yeast *URA3* gene, and a fragment containing two inverted 280 bp $C_{1-3}A/TG_{1-3}$ tracts separated by the kanamycin-resistance gene. In addition, the *ARSH4* yeast origin of replication on a 380 bp *Sma*I–*Hin*cII fragment of pAB9 (25) was inserted either next to the *URA3* gene (for YRpRW40), or next to the *LEU2* gene (for YRpRW41) (Fig. 1). Digestion of either YRpRW40 or YRpRW41 with *Bam*HI produces a 9 kb linear fragment with a tract of 280 bp of $C_{1-3}A/TG_{1-3}$ sequences at each end. These linear vectors, named YLpRW40 and YLpRW41, respectively (Fig. 1, bottom), were used to transform yeast cells, and Leu^+/Ura^+ cells were analyzed for correct structure of the plasmids by Southern blotting (data not shown; Fig. 2).

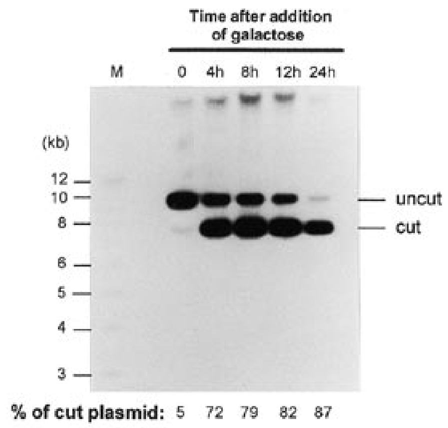

Figure 2. Plasmid cleavage *in vivo*. Autoradiograph of a Southern blot analysis showing *HO*-mediated *in vivo* cutting of YLpRW41 in RWY100 cells. Low molecular weight DNA was extracted via a Hirt procedure from strain RWY100 at the following indicated time points: 0, no galactose added; 4, 8, 12 and 24 h, cells that were harvested after 4, 8, 12 or 24 h of galactose addition. Lane M contains end-labeled molecular weight marker DNA. Undigested DNA was subjected to agarose gel electrophoresis and blotted to a nylon membrane. The blot was hybridized to a *LEU2* probe. The positions in the gel of the 9 kb uncut DNA fragment and the 7.4 kb *HO*-cut fragment are indicated. Percentage of cut plasmids was determined by scanning the blot using a PhosphorImager and is indicated (bottom).

In order to insert a galactose inducible *HO* gene into the yeast genome, pA2XbHO was constructed in the following way: an 850 bp *Eco*RI–*Stu*I fragment of YEpFAT10 (26) harboring the *TRP1* gene was ligated to a 8.8 kb *Sph*I–*Sma*I fragment of YCpHOCUT4 (24) containing the yeast *HO* endonuclease gene under the control of the *GAL10* promoter, to yield YCpGHOTRPII. A 5.0 kb *Eco*RI–*Spe*I fragment of YCpGHOTRPII was then cloned into *Eco*RV–*Stu*I digested pA2Xb to form pA2XbHO.

pA2Xb was pVZ1 (27) into which was cloned a 1 kb *Xba*I–*Xba*I fragment derived from the *ADE2* gene.

A 5.4 kb *Eco*RI–*Sal*I fragment of pA2XbHO which contains the inducible *HO* gene and the *TRP1* gene, flanked by appropriate sequences of the *ADE2* locus, was then used to transform yeast strain AR120 (*Mat**a***, *cdc7*, *bar1*, *ura3-52*, *his6*, *trp1-289*, *leu2-3, 112*, *HML**a***, *HMR**a***) (24) to yield RWY100, using the one-step gene replacement technique (28). Proper insertion of the fragment in the desired locus was ascertained by Southern analysis (data not shown), and by the fact that the RWY100 cells form red colonies due to the disruption of the *ADE2* locus (29).

Cloning in bacteria used standard *Escherichia coli* strains and growth conditions (30). Yeast growth media were as described previously (31), and yeast transformations were performed using a modified LiAc method (32,33).

Cell synchronization and DNA isolation

RWY100 cells containing either YLpRW40 or YLpRW41 were grown in Yc–Ura–Leu media containing glycerol (2%) and lactate (2%) and synchronized using two consecutive blocks (α-factor and *cdc7*), essentially as described previously (13,14,34). Briefly, to arrest cells in G_1-phase, α-factor was added to a non-synchronously growing culture and the cells incubated for 12 h. Galactose (2% final concentration) was then added to induce expression of the *HO* endonuclease and the cells were incubated for 5 h. Subsequently, glucose was added to repress *HO* gene expression (1 h). The cells were then shifted to 37°C, the restrictive temperature for the cdc7 mutation, and incubated for 4 h. Cells were then released into S-phase by a return to the permissive temperature. Total genomic and plasmid DNA was isolated using a modified glass bead procedure (14,35), or a Hirt procedure designed to isolate low molecular weight DNA (36).

DNA analysis

One- and two-dimensional agarose gel techniques, Southern blotting and hybridization conditions were described previously (14,34). Note that the non-denaturing Southern procedure used in Figure 4A and B only yields reliable signals for relatively small DNA fragments (<3–4 kb) (37). Probes for hybridization were obtained by a random priming labeling procedure (38). DNAs used were a 1 kb *Xho*I–*Bam*HI fragment of λ DNA (nt 33498–34500), a 300 bp fragment containing 280 bp of telomeric repeats derived from pYLPV (14), a 1.3 kb *Ssp*I–*Ssp*I fragment derived from the *LEU2* gene in pRS305 (22), and a 900 bp *Nsi*I–*Nde*I fragment derived from the *URA3* gene in pRS306 (22).

Quantification of the radioactivity in signals was by storage phosphorimaging using the Molecular Dynamics PhosphorImager™ SF with the MD ImageQuant software (version 3.3) (39). A background value for an area of equal size was obtained for each lane and subtracted from the signal.

RESULTS

Plasmid cleavage *in vivo*

In order to establish two identical linear plasmids which only differed in that one did and the other did not contain an origin of replication, we took advantage of the properties of the *HO* endonuclease. If an appropriate recognition site is present, the *HO* endonuclease will create a double-strand break in the DNA (23,40). Thus, the *HO* endonuclease gene under control of the *GAL10* promoter was inserted in the genome of the strain AR120 (24), yielding RWY100. The two linear plasmids YLpRW40 and YLpRW41 (Fig. 1) were then introduced into RWY100 cells grown on glucose-containing media. Both of these linear constructs contain an internal tract of telomeric sequences with an *HO* recognition sequence at the distal ends (Fig. 1). Upon shift of these cells into galactose media, transcription of the *HO* gene is activated and cuts the two linear plasmids. The resulting linear fragments will have an established normal telomere without any exogenous sequences at one end, and a tract of 280 bp of telomeric sequences followed by ~50 bp non-telomeric sequences before the *HO* cut at the other end. Most importantly, the two plasmids will differ in that the fragment derived from YLpRW41 contains an origin of replication and the fragment derived from YLpRW40 does not, because the origin of replication used to establish the plasmid was located on the fragment distal to the *HO*-cut site and thus lost after *HO* induction (Fig. 1).

While such *HO*-induced chromosome fragmenting has been successfully used by others (24,41,42), it was important to assess the efficiency of plasmid cutting by the *HO* endonuclease in our particular constructs. RWY100 yeast cells transformed with YLpRW41 were first grown in synthetic medium containing lactate and glycerol. *HO* gene expression was induced by the addition of galactose, and DNA was prepared and analyzed for efficiency of *HO* cutting from samples taken at various times thereafter (Fig. 2). Four hours after galactose addition, >70% of the plasmids YLpRW41 were converted to the shortened form and more prolonged incubation times only yielded a slight increase beyond that level (Fig. 2). Virtually identical results were obtained for the linear plasmid YLpRW40 (data not shown). Thus, we chose to use 5 h of galactose-induced plasmid cleavage for the following experiments.

The linear plasmid without an origin of replication does not replicate

Since we wished to analyze and compare the behavior of the telomeres on replicating and non-replicating linear DNA plasmids, it was important to establish that the used constructs indeed behaved as required. Replication intermediates indicating active replication on a DNA fragment can be detected by 2D agarose gel electrophoresis (34). A DNA fragment with an asymmetrically placed replication origin such as the ones expected on the uncut YLpRW41 and YLpRW40 yield a 'bubble-to-Y' pattern of replication intermediates. When DNA isolated from RWY100 cells carrying YLpRW41 was analyzed for replication intermediates, such a 'bubble-to-Y' pattern was detected (Fig. 3A, right). In an identical analysis on DNA derived from cells containing plasmid YLpRW40, only the 'Y' portion of pattern was detected and the small 'bubble' arc was missing (Fig. 3A, left). However, it must be noted that in order to detect a full 'bubble-to-Y' pattern for any given linear DNA molecule, the actual origin must be located in the central third of the molecule. If the origin is located on the outer third of the fragment on either side, the very small 'bubbles' remain undetectable and only the 'Y' portion of the pattern is visible (43,44). Since the origin on YLpRW40 is in fact very close to the end of the molecule (Fig. 1), it is not surprising that the 'bubble' part was not detectable in our experiments and the pattern seen in Figure 3A (left) corresponds to the expected

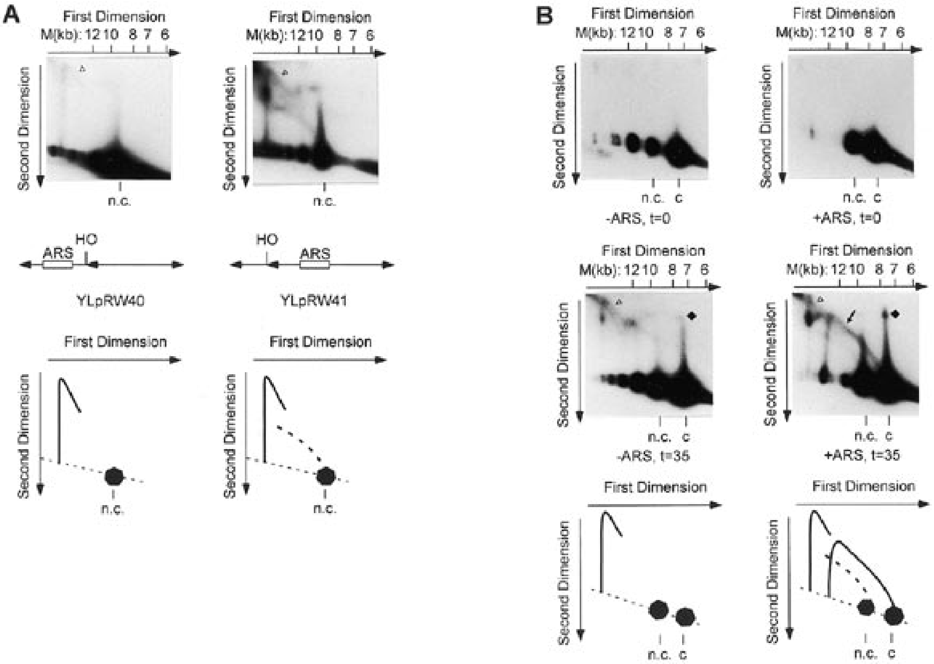

Figure 3. Replication intermediates (RIs) of YLpRW40 and YLpRW41 as detected by 2D agarose gel electrophoresis. (**A**) Analysis of DNA derived from RWY100 cells containing uncut linear plasmids YLpRW40 (left) or YLpRW41 (right). 2D gels using these DNAs were performed using standard procedures (34) and hybridized to a λ probe (left gel) or a *URA3* probe (right gel). Below the gels, a simplified diagram of the plasmids with the relative placements of the origins, and a drawing representing the 2D-gels are shown. Δ, the 'Y' portions of the replication intermediates. Note that due to the closeness of the *ARSH4* to the end on plasmid YLpRW40, no 'bubble' arc is detectable for this plasmid (see text). (**B**) Total yeast DNA was isolated from cells containing YLpRW40 (–ARS) or YLpRW41 (+ARS), which were either arrested in G_1-phase with α-factor and induced with galactose for 5 h (top, t = 0), or which were released into a synchronous S-phase for 35 min. (middle, t = 35) (see Materials and Methods for the arrest and release protocol). 2D gels were as in (A) and the blots were hybridized to the λ probe. M, molecular size standards (in kb) run in a parallel lane in the first dimension. n.c., non-cut plasmid; c, cut plasmid; Δ, RIs of the non-cut 9 kb plasmids [as in (A)]; ➞, RIs of the cut 7.4 kb plasmid; ◆, CFP (circular form of the plasmid) of the cut plasmids. Simplified diagrams of the RIs detected on the middle gels are depicted on the bottom part.

pattern for such a fragment. Thus, the analysis of the replication intermediates for YLpRW40 and YLpRW41 yielded the expected patterns on the 2D gels, indicating that the *ARSH4* placed on the plasmids was used as origin of replication. Next, we analyzed the replication intermediates of the linear plasmids after *HO*-induced cutting. Cells containing YLpRW40 or YLpRW41 were synchronized at the G_1–S boundary using a two-step procedure (Materials and Methods). The *HO* endonuclease was induced by addition of galactose in α-factor arrested cells and, after 5 h, the cells were shifted to 37°C, the restrictive temperature for the *cdc7* allele. Finally, cells were released into S-phase by a return to the permissive temperature (23°C) for *cdc7*. DNA was prepared from G_1-arrested and S-phase cells (35 min after the release) and examined by 2D gel electrophoresis (Fig. 3B). As expected, when the filters with DNA derived from G_1 cells were hybridized with a λ probe, no replication intermediates were detected for either plasmid (Fig. 3B, top panels). When the control plasmid YLpRW41 derived from S-phase cells was analyzed, a strong 'bubble-to-Y' arc was observed (arrow in Fig. 3B, middle right). As the signal for the double-stranded linear molecule migrated as a 7.4 kb fragment, this arc clearly emanated from the molecules that were cut by the *HO* endonuclease. Since ~75% of the molecules were cut by *HO*, this arc yields a much stronger signal than the arc derived from the small proportion of the plasmid YLpRW41 that was not cut. Nevertheless, a weak 'bubble-to-Y' pattern, which was virtually identical to the pattern observed in Figure 3A for the uncut plasmid, was discernible (open triangles in Fig. 3). For the plasmid YLpRW40 that was cut by the *HO* endonuclease, there were no replication intermediates emanating from the cut fragment detectable (Fig. 3B, middle left). However, the remaining uncut 9 kb plasmid YLpRW40 yielded a weak 'Y' pattern of replication intermediates, as expected and observed in Figure 3A. As additional controls for the replication behavior of the plasmids, cells in which the endonuclase *HO* had been induced were subsequently plated on synthetic media lacking leucine and containing 5-fluoro-orotic acid (5-FOA). On such plates, only cells that contained *HO*-cut shortened plasmids without the *URA3* gene are expected to grow (45). If the fragments derived from the input linear plasmids can replicate, strains containing this shortened fragment should be able to grow. While strains containing only the *HO*-cut shortened fragment derived from YLpRW41 were easily recovered, strains containing the *HO*-cut YLpRW40 fragment gave only very few colonies on the YC–Leu+FOA plates (data not shown). When the DNA of those strains that did grow was analyzed, only rearranged plasmids, or plasmids that had integrated into the genome were found. No clone contained the cut YLpRW40 fragment as a free linear plasmid (data not shown). In addition, a circular 7 kb plasmid which contained two telomeric tracts, the *LEU2* gene and λ sequences but no ARS did not yield transformants with an episome (data not shown). Taken together, these data show that the linear 7 kb fragment left after *HO*-mediated cutting of plasmid YLpRW40 is unable to sustain efficient replication, whereas the 7.4 kb fragment derived from YLpRW41and containing the *ARSH4* replicates efficiently.

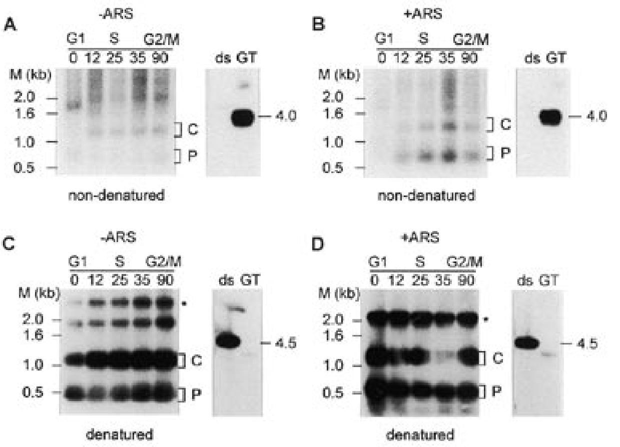

Figure 4. G-tails are only present on TRFs of a replicating plasmid. (**A** and **B**) Non-denaturing Southern blots prepared from synchronized cells. Total DNA was isolated from strain RWY100 containing YLpRW40 (–ARS) or YLpRW41 (+ARS) at the following time points: 0, cells arrested in G_1 with α-factor and induced with 2% galactose for 5 h; 12, 25, 35 and 90, cells were released into a synchronous S-phase for 12, 25, 35 or 90 min, respectively. M, molecular weight standards. The DNAs were digested with the restriction enzyme *Xho*I and separated on a 0.6% agarose gel, which was then processed as a non-denaturing Southern blot and hybridized to a ^{32}P-labeled telomeric repeat probe (14). Above the actual time points, the corresponding approximate cell cycle phases are indicated. Lanes ds and GT contain linearized double-stranded pMW55 DNA and single-stranded phagemid DNA derived from pGT75, respectively (16). (**C** and **D**) Duplicates of the gels presented in (A) and (B) respectively were prepared as standard denaturing Southern blots and hybridized to the same probe as (A) and (B). C, chromosomal telomeres; P, plasmid telomeres. Asterisks indicate the terminal *Xho*I restriction fragments derived from the *HO*-uncut plasmids YLpRW40 (2.6 kb, left panel) and YLpRW41 (2.1 kb, right panel).

The exonuclease activity is dependent on replication fork passage

The above results strongly suggest that on the *HO*-cut fragments derived from YLpRW41, initiation of replication occurred efficiently, while on the corresponding fragments derived from YLpRW40, virtually no initiation occurred. During telomere replication, single-stranded TG_{1-3} DNA appears at the end of S-phase (15,16). To examine whether the activity required to produce G-tails is dependent on a passage of the regular replication machinery or whether they are generated by a cell-cycle-regulated process that is independent of the actual replication of telomeres, we used the RWY100 strain containing either YLpRW40 or YLpRW41. The cells were synchronized as described above; total DNA derived from cells at different time points after the release into S-phase was then digested with *Xho*I and analyzed by non-denaturing Southern hybridization (14). Most terminal restriction fragments (TRFs) for genomic DNA digested with *Xho*I are ~1.3 kb in size due to a conserved *Xho*I site in the yeast subtelomeric Y′ element that is present at most telomeres (46). TRFs generated by *Xho*I digestion and shorter than 1.3 kb are not observed for genomic DNA. The linear plasmids YLpRW40 and YLpRW41 were constructed such that after the *HO*-induced cleavage, both TRFs derived from these plasmids were ~600 bp (Fig. 1), and thus clearly distinct from any genomic TRFs. For cells arrested in G_1 there was no signal for chromosomal telomeres nor for telomeres of the plasmids (Fig. 4A and B, lanes 0, and Fig. 5). In DNA derived from cells containing either YLpRW40 or YLpRW41, there was a signal at ~1.3 kb corresponding to G-tails on the chromosomal TRFs for cells in S-phase (Figs 4A and B and 5). We also detected G-tails in S-phase on TRFs of the replicating plasmid at 600 bp (Figs 4B and 5). It was shown previously that a linear plasmid forms telomere–telomere associations that are dependent on the presence of TG_{1-3} tails (14). Those circular forms of the plasmid (CFP) can be detected by 2D gel electrophoresis (14). Thus, consistent with the G-tail analysis, a CFP was detected for the replicating fragment (Fig. 3B, middle right, closed diamond), but remained virtually undetectable for the non-replicating fragment. As expected, the strongest signal for G-tails in cells containing the plasmid YLpRW41 was at 35 min after the release into S-phase (13,14) (Fig. 5, top panel). The strongest signal for G-tails on genomic TRFs derived from cells containing the non-replicating plasmid was at 12 min after the release (Fig. 5, bottom). We do not know whether this difference in the time of G-tail detection on chromosomal TRFs is due to the presence of the replicating plasmid or whether it is a simple experimental variation. It is known that individual synchrony experiments may vary considerably in their absolute timing of replication after the release (13,47). Most importantly, however, we did not detect significant G-tail signals for YLpRW40-plasmid derived TRFs at any time point (Figs 4A and 5). The same DNA as shown in Figure 4A and B was then analyzed by regular denaturing Southern hybridization (Fig. 4C and D). The TRFs derived from the plasmid YLpRW40 were clearly detectable and yielded bands of very similar intensity to those derived from the plasmid YLpRW41, indicating that there were comparable quantities of DNA for the two plasmids in our analysis (Fig. 4, compare C and D).

It could be argued that the ends of the linear plasmids created by the *HO* endonuclease contained some 50 bp of non-telomeric DNA, precluding a proper telomere processing and detection in our non-denaturing Southern assay. However, it is important to realize that in each case, the end opposite to the *HO*-cut was an *in vivo* established telomere, which we presume had a natural end-structure. Thus, at least this end (half of all plasmid-born telomeres) is expected to be in a natural configuration, and if G-tails were generated only on this end, they would have been detected as were the G-tails for the TRFs derived from *HO*-cut YLpRW41. Taken together, the data thus demonstrate that G-tail formation on plasmid telomeres only occurs if the plasmid is replicating.

DISCUSSION

Maintaining a functional tract of telomeric repeats is essential for chromosome stability in eukaryotic cells (1,9,48). It is thought that the actual length of the repeats will be determined by complex mechanisms that include shortening as well as lengthening activities. Much recent effort has been placed to elucidate the regulation of the enzyme telomerase, but it is also clear that this enzyme will only be able to synthesize telomeric repeats of the G-rich strand (8). The C-rich strand is generally believed to be synthesized by the polα–primase complex, but there is very little direct evidence for this notion (reviewed in 49). One study proposed a telomere-specific primase, synthesizing telomeric repeats of the C-rich strand for the initiation of replication on the gene-sized linear

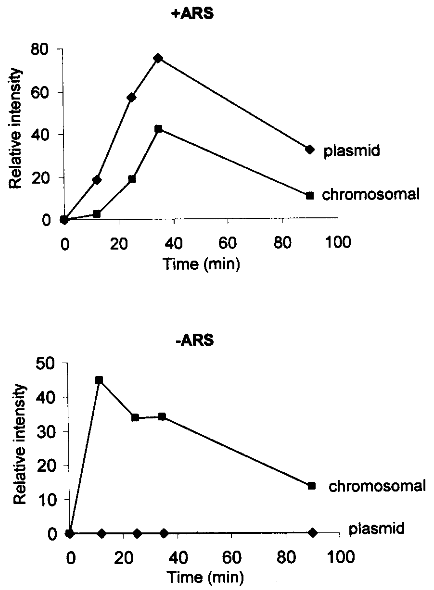

Figure 5. Quantification of TG_{1-3} tails on chromosomal Y′ telomeres and telomeres of *HO*-cut plasmids YLpRW40 (–ARS) and YLpRW41 (+ARS). The relative intensities of the signals shown in Figure 4A and B were determined using a PhosphorImager and corrected for DNA loading using gels shown in Figure 4C and D. Relative intensity (arbitrary units) is plotted with respect to the number of minutes after release into S-phase.

DNA fragments in the *Oxytricha nova* macronucleus (50). However, such a mode of replication initiation appears not to occur in many organisms since at least in yeast, linear DNA fragments with telomeric ends but without an internal origin of replication are not replicated (51; this study). A possible coordinate regulation of the C- and G-rich strands has been inferred from studies in *Euplotes crassus*, in which an inhibition of DNA polymerase activity by aphidicolin resulted in abnormal lengths of the C- and G-strands (52). Consistent with this idea, telomerase RNA was found to colocalize with the regular replication machinery in specific subnuclear compartments in ciliates (53).

For yeast, it was demonstrated that mutations affecting a number of proteins that are part of the replication fork machinery lead to abnormal telomeric repeat lengths. For instance, mutations affecting the catalytic subunit of polymerase α (*CDC17*/*POL1*) or mutations in the large subunit of replication factor C (*CDC44*/*RFC1*) yield elongated telomeres when the strains are grown at semi-permissive temperatures (54,55). This elongation is dependent on an active telomerase, suggesting that there exists a cross-regulation between telomerase and the replication fork (55). In addition, yeast telomeres acquire long G-tails at the end of S-phase, after conventional replication is virtually complete (13,14). The single-stranded extensions are the product of at least two processes, namely a shortening of the C-strand due to an exonuclease and a lengthening due to telomerase (15,16). Since these G-tails will provide the template for C-strand synthesis, the activities involved in their generation may provide a means of regulating the extent of double-stranded telomeric repeats that are maintained. For example, in yeast strains devoid of telomerase, the G-tails observed at the end of S-phase are presumably generated by C-strand resection and no net elongation can occur (15). Therefore, due to the end-replication problem during the fill-in process on the G-tails, the telomeres shorten by ~5 bp/generation in such strains (10). On the other hand, C-strand fill-in synthesis may regulate the extent of G-strand elongation. It has been hypothesized that defects in the replication machinery needed to fill in the G-tails may disrupt a negative regulation of G-tail elongation by telomerase (55).

In order to obtain further insights into the precise events taking place during telomere replication in yeast, we have started to investigate the nature of the C-strand recessing activities. We show here that C-strand resection on plasmid telomeres is dependent on the passage of a replication fork. On a linear DNA fragment without a bona fide ARS element, no G-tails can be detected in S-phase and no telomere–telomere associations are formed (Figs 3B, 4A and 5). Conversely, an equivalent construct containing an ARS acquires G-tails in late S-phase and circular forms of the plasmid are detectable (Figs 3B, 4B and 5). We conclude that the generation of G-tails on plasmid telomeres is dependent on the passage of the conventional replication machinery. These results exclude the possibility that the C-strand recessing occurs in a replication-independent fashion and suggest that the activities responsible for this telomere processing are associated with the conventional replication machinery. Alternatively, the chromosome ends may be protected from this exonuclease activity by the presence of end-binding factors such as the Ku proteins (20), which may be associated with chromosome ends throughout the cell cycle. Consistent with this hypothesis, in cells lacking Ku, chromosome ends display G-tails constitutively (20).

These results suggest a model for the consecutive steps involved in telomere processing in yeast. In the G_1 phase of the cell cycle, the ends of chromosomes are bound by a terminal protein complex and the lengths of the repeats as well as the terminal DNA structure are stable. After initiation of DNA replication at chromosome internal sites, the replication fork machinery passes through the telomeric DNA, displacing the terminal complex. At this stage, the ends can become accessible to exonucleolytic attack degrading the 5′ ends and long G-tails are generated. It remains possible that this resectioning of the 5′ ends only occurs on ends replicated by leading-strand synthesis. On ends replicated by lagging strand synthesis, priming could stop at a distance from the actual ends, resulting in such G-tails without the necessity of additional processing by an exonuclease. The G-tails are then substrates for telomerase mediated elongation and for fill-in synthesis by polα–primase. Rebinding of the terminal complex and the setup of a heterochromatin-like domain would preclude further lengthening or shortening of the telomeric repeats and the ends would be stable until the next round of replication. In support of this model, genes placed in the vicinity of telomeres can switch from a repressed to a derepressed state only after replication is virtually complete and before the next G_1 phase (56). In G_0, G_1 and early S-phase, at least a subset of transcriptional transactivators cannot overcome the silencing imposed by the telomeric chromatin domain and derepression requires the passage of a replication fork (56).

It will be of interest to know which gene product(s) are responsible for the resection of the C-rich strand at telomeres. Searching the literature for known yeast exonucleolytic activities and using an alignment of the identified sequence motifs in known 5′–3′ exonucleases with the yeast genome, we identified a number of candidate genes for this activity (57–59; and data not shown). However, yeast strains carrying deletions of either *EXO1*, *RAD17*, *RAD24*, *RNC1*/*NUC2* or *YER041W* did not show any differences in G-tail formation or telomere length when compared with wild-type strains (I.Dionne, S.Gravel and R.J.Wellinger, unpublished data). The *RAD50*/*MRE11*/*XRS2* complex has also been proposed to have exonucleolytic activity (60–62), and recently it was shown that yeast strains with mutations in either of these genes display shortened telomeric repeat tracts (63,64). These and other data have led to the proposal that this complex is the exonuclease responsible for C-strand processing (65). However, when chromosomal DNAs derived from cells carrying deletions of the *MRE11* or the *RAD50* gene were analyzed for their terminal DNA configurations, no differences between these DNAs and DNAs derived from isogenic wild-type strains were observed (I.Dionne and R.J.Wellinger, unpublished).

Since maintaining telomeric repeats is an essential requirement for the stability of eukaryotic chromosomes, a comprehension of all the factors involved in these mechanisms will be of utmost importance to our understanding of chromosome replication and function. We show here that an activity that processes telomeric C-strands is either associated with the conventional replication machinery or only gains access to chromosome ends after disruption of the terminal protein–DNA complex by the passage of a replication fork. Genetic and biochemical approaches should now allow an identification of the presumed exonuclease and the study of its regulation will shed new light into the mechanisms governing telomere maintenance in yeast and mammals.

ACKNOWLEDGEMENTS

We thank M. K. Raghuraman, W. L. Fangman and M. M. Smith for generous gifts of plasmids and yeast strains. This research was supported by a grant from the Medical Research Council of Canada (Grant MT 12616). I.D. is supported by a studentship from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR). R.J.W. is a Chercheur-Boursier Senior of the Fonds de la Recherche en Santé du Québec (FRSQ).

REFERENCES

1 Zakian,V.A. (1996) *Annu. Rev. Genet*., **30**, 141–172.
2 Wellinger,R.J. and Sen,D. (1997) *Eur. J. Cancer*, **33**, 735–749.
3 Klobutcher,L.A., Swanton,M.T., Donini,P. and Prescott,D.M. (1981) *Proc. Natl Acad. Sci. USA*, **78**, 3015–3019.
4 Henderson,E.R. and Blackburn,E.H. (1989) *Mol. Cell. Biol*., **9**, 345–348.
5 Makarov,V.L., Hirose,Y. and Langmore,J.P. (1997) *Cell*, **88**, 657–666.
6 McElligott,R. and Wellinger,R.J. (1997) *EMBO J*., **16**, 3705–3714.
7 Lingner,J., Cooper,J.P. and Cech,T.R. (1995) *Science*, **269**, 1533–1534.
8 Greider,C.W. (1996) *Annu. Rev. Biochem*., **65**, 337–365.
9 Lundblad,V. and Szostak,J.W. (1989) *Cell*, **57**, 633–643.
10 Singer,M.S. and Gottschling,D.E. (1994) *Science*, **266**, 404–409.
11 Greider,C.W. and Blackburn,E.H. (1985) *Cell*, **43**, 405–413.
12 Shampay,J., Szostak,J.W. and Blackburn,E.H. (1984) *Nature*, **310**, 154–157.
13 Wellinger,R.J., Wolf,A.J. and Zakian,V.A. (1993) *Mol. Cell. Biol*., **13**, 4057–4065.
14 Wellinger,R.J., Wolf,A.J. and Zakian,V.A. (1993) *Cell*, **72**, 51–60.
15 Wellinger,R.J., Ethier,K., Labrecque,P. and Zakian,V.A. (1996) *Cell*, **85**, 423–433.
16 Dionne,I. and Wellinger,R.J. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 13902–13907.
17 Gottschling,D.E. and Zakian,V.A. (1986) *Cell*, **47**, 195–205.
18 Price,C.M. (1990) *Mol. Cell. Biol*., **10**, 3421–3431.
19 Sheng,H., Hou,Z., Schierer,T., Dobbs,D.L. and Henderson,E. (1995) *Mol. Cell. Biol*., **15**, 1144–1153.
20 Gravel,S., Larrivée,M., Labrecque,P. and Wellinger,R.J. (1998) *Science*, **280**, 741–744.
21 Bertuch,A. and Lundblad,V. (1998) *Trends Cell Biol*., **8**, 339–342.
22 Sikorski,R.S. and Hieter,P. (1989) *Genetics*, **122**, 19–27.
23 Kostriken,R., Strathern,J.N., Klar,A.J., Hicks,J.B. and Heffron,F. (1983) *Cell*, **35**, 167–174.
24 Raghuraman,M.K., Brewer,B.J. and Fangman,W.L. (1994) *Genes Dev*., **8**, 554–562.
25 Bouton,A.H. and Smith,M.M. (1986) *Mol. Cell. Biol*., **6**, 2354–2363.
26 Conrad,M.N., Wright,J.H., Wolf,A.J. and Zakian,V.A. (1990) *Cell*, **63**, 739–750.
27 Henikoff,S. and Eghtedarzadeh,M.K. (1987) *Genetics*, **117**, 711–725.
28 Rothstein,R.J. (1983) *Methods Enzymol*., **101C**, 202–211.
29 Roman,H. (1956) *Cold Spring Harbor Symp. Quant. Biol*., **21**, 175–185.
30 Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*, 1st edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
31 Rose,M.D., Winston,F. and Hieter,P. (1990) *Methods in Yeast Genetics: A Laboratory Course Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
32 Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) *J. Bacteriol*., **153**, 163–168.
33 Gietz,R.D. and Schiestl,R.H. (1995) *Meth. Mol. Cell. Biol*., **5**, 255–269.
34 Brewer,B.J. and Fangman,W.L. (1987) *Cell*, **51**, 463–471.
35 Huberman,J.A., Spotila,L.D., Nawotka,K.A., El-Assouli,S.M. and Davis,L.R. (1987) *Cell*, **51**, 473–481.
36 Livingston,D.M. and Kupfer,D.M. (1977) *J. Mol. Biol*., **116**, 249–260.
37 Wellinger,R.J., Wolf,A.J. and Zakian,V.A. (1992) *Chromosoma*, **102**, S150–S156.
38 Feinberg,A.P. and Vogelstein,B. (1983) *Anal. Biochem*., **132**, 6–13.
39 Johnston,R.F., Pickett,S.C. and Barker,D.L. (1990) *Electrophoresis*, **11**, 355–360.
40 Herskowitz,I. and Jensen,R.E. (1991) *Methods Enzymol*., **194**, 132–146.
41 Kramer,K.M. and Haber,J.E. (1993) *Genes Dev*., **7**, 2345–2356.
42 Sandell,L.S. and Zakian,V.A. (1993) *Cell*, **75**, 729–739.
43 Krysan,P.J. and Calos,M.S. (1991) *Mol. Cell. Biol*., **11**, 1464–1472.
44 Linskens,M.H.K. and Huberman,J.A. (1990) *Nucleic Acids Res*., **18**, 647–652.
45 Boeke,J.D., LaCroute,F. and Fink,G.R. (1984) *Mol. Gen. Genet*., **197**, 345–346.
46 Chan,C.S.M. and Tye,B.-K. (1983) *J. Mol. Biol*., **168**, 505–523.
47 McCarroll,R.M. and Fangman,W.L. (1988) *Cell*, **54**, 505–513.
48 Lee,H.-W., Blasco,M.A., Gottlieb,G.J., Horner,J.W., Greider,C.W. and DePinho,R.A. (1998) *Nature*, **392**, 569–574.
49 Price,C.M. (1997) *Biochemistry (Moscow)*, **62**, 1216–1223.
50 Zahler,A.M. and Prescott,D.M. (1989) *Nucleic Acids Res*., **17**, 6299–6317.
51 Wellinger,R.J. and Zakian,V.A. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 973–977.
52 Fan,X. and Price,C.M. (1997) *Mol. Biol. Cell*, **8**, 2145–2155.
53 Fang,G. and Cech,T.R. (1995) *J. Cell. Biol*., **130**, 243–253.
54 Carson,M.J. and Hartwell,L. (1985) *Cell*, **42**, 249–257.
55 Adams,A.K. and Holm,C. (1996) *Mol. Cell. Biol*., **16**, 4614–4620.
56 Aparicio,O.M. and Gottschling,D.E. (1994) *Genes Dev*., **8**, 1133–1146.
57 Tishkoff,D.X., Boerger,A.L., Bertrand,P., Filosi,N., Gaida,G.M., Kane,M.F. and Kolodner,R.D. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 7478–7492.
58 Lydall,D. and Weinert,T. (1995) *Science*, **270**, 1488–1491.
59 Moore,P.D., Simon,J.R., Wallace,L.J. and Chow,T.Y.-K. (1993) *Curr. Genet*., **23**, 1–8.
60 Nairz,K. and Klein,F. (1997) *Genes Dev*., **11**, 2272–2290.
61 Ivanov,E.L., Sugawara,N., White,C.I., Fabre,F. and Haber,J.E. (1994) *Mol. Cell. Biol*., **14**, 3414–3425.
62 Tsubouchi,H. and Ogawa,H. (1998) *Mol. Cell. Biol*., **18**, 260–268.
63 Kironmai,K.M. and Muniyappa,K. (1997) *Genes Cells*, **2**, 443–455.
64 Boulton,S.J. and Jackson,S.P. (1998) *EMBO J*., **17**, 1819–1828.
65 Nugent,C.I., Bosco,G., Ross,L.O., Evans,S.K., Salinger,A.P., Moore,J.K., Haber,J.E. and Lundblad,V. (1998) *Curr. Biol*., **8**, 657–660.