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Exo1 and Rad24 Differentially Regulate Generation of ssDNA at Telomeres of *Saccharomyces cerevisiae cdc13-1* Mutants

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ABSTRACT

Cell cycle arrest in response to DNA damage depends upon coordinated interactions between DNA repair and checkpoint pathways. Here we examine the role of DNA repair and checkpoint genes in responding to unprotected telomeres in budding yeast *cdc13-1* mutants. We show that Exo1 is unique among the repair genes tested because like Rad9 and Rad24 checkpoint proteins, Exo1 inhibits the growth of *cdc13-1* mutants at the semipermissive temperatures. In contrast Mre11, Rad50, Xrs2, and Rad27 contribute to the vitality of *cdc13-1* strains grown at permissive temperatures, while Din7, Msh2, Nuc1, Rad2, Rad52, and Yen1 show no effect. Exo1 is not required for cell cycle arrest of *cdc13-1* mutants at 36° but is required to maintain arrest. Exo1 affects but is not essential for the production of ssDNA in subtelomeric Y′ repeats of *cdc13-1* mutants. However, Exo1 is critical for generating ssDNA in subtelomeric X repeats and internal single-copy sequences. Surprisingly, and in contrast to Rad24, Exo1 is not essential to generate ssDNA in X or single-copy sequences in *cdc13-1 rad9*Δ mutants. We conclude that Rad24 and Exo1 regulate nucleases with different properties at uncapped telomeres and propose a model to explain our findings.

CHECKPOINT controls are evolutionarily conserved mechanisms that inhibit cell cycle progression when DNA is damaged (Hartwell and Weinert 1989; Lowndes and Murguia 2000; Zhou and Elledge 2000; Nyberg *et al.* 2002). They play important roles in the processes of meiosis and immune system development, contribute to the integrity of the neuronal system, help to maintain genetic stability, and prevent cancer (Zhou and Elledge 2000). Checkpoint pathways are thought of as signal transduction cascades that comprise stimuli, sensors, signalers, and targets (Zhou and Elledge 2000, 2003; Nyberg *et al.* 2002).

Recently two checkpoint sensor protein complexes have been shown to bind damaged DNA (Kondo *et al.* 2001; Melo *et al.* 2001; Rouse and Jackson 2002; Zou *et al.* 2002). In budding yeast, one complex comprises Rad17, Mec3, and Ddc1, forming a heterotrimeric, proliferating cell nuclear antigen (PCNA)-like ring structure, called the 9-1-1 complex (named after the mammalian and *Schizosaccharomyces pombe* orthologs Rad9, Rad1, and Hus1). Loading of this complex is dependent on an alternative replication factor C (RFC) complex made of Rad24 and the four small Rfc subunits (Green *et al.* 2000). The second, Mec1/Ddc2 complex, binds DNA independently of Rad24, Rad17, Mec3, and Ddc1. Both Rad17 and Mec1 complexes are essential for signaling cell cycle arrest in response to many types of DNA damage, suggesting that they are each necessary to stimulate the signal transduction cascade that results in cell cycle arrest. Another checkpoint protein, Rad9, is required to load neither the Rad17 nor the Mec1 complex and it may therefore act as a downstream signal transduction molecule or as a component of a third checkpoint complex (Gilbert *et al.* 2001; Melo *et al.* 2001). The nature of the interactions between checkpoint sensor proteins and damaged DNA is now being elucidated (Ellison and Stillman 2003; Majka and Burgers 2003; Zou and Elledge 2003).

A large body of evidence indicates that single-stranded DNA (ssDNA) is an important stimulus for cell cycle arrest in eukaryotes (Garvik *et al.* 1995; Huang *et al.* 1996; Lee *et al.* 1998; Usui *et al.* 2001; Vaze *et al.* 2002; Zou and Elledge 2003). Interestingly, checkpoint proteins not only recognize ssDNA but affect the rate at which ssDNA arises, suggesting that they have direct roles in regulating accumulation of ssDNA (Lydall and Weinert 1995).

Cells that are defective in Cdc13, a telomere-binding protein, accumulate large amounts of ssDNA specifically near telomeres (Garvik *et al.* 1995; Nugent *et al.* 1996; Booth *et al.* 2001). *rad9*Δ and *rad24*Δ checkpoint mutants are completely defective in cell cycle arrest in response to *cdc13-1*-induced defects, yet Rad24 contributes

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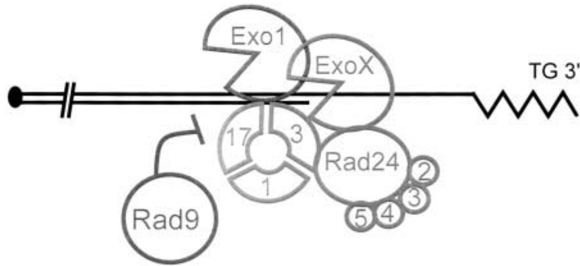

Figure 1.—A model of checkpoint regulation of Exo1 activity at *cdc13-1* telomeres. This model implies that Rad24 and the small Rfc subunits (2, 3, 4, 5) load the checkpoint sliding clamp (Ddc1, Mec3, and Rad17) onto telomeres of *cdc13-1* mutants at 36°, and this sliding clamp tethers Exo1 to DNA. Other nuclease activities (ExoX) may also exist. Rad9 inhibits nuclease activity.

to ssDNA production, while Rad9 inhibits ssDNA production (Lydall and Weinert 1995). These observations can be explained by a model in which Rad24 is required for the activity of a 5′ to 3′ exonuclease that degrades the telomeres of Cdc13 mutants and in which Rad9 inhibits this putative exonuclease (Booth *et al.* 2001). One model is that the PCNA-like 9-1-1 complex loaded onto DNA by Rad24/Rfc possesses intrinsic exonuclease activity. This is plausible because there is evidence that members of the 9-1-1 complex possess 3′ to 5′ exonuclease activity *in vitro* (Freire *et al.* 1998; Naureckiene and Holloman 1999; Bessho and Sancar 2000; Lindsey-Boltz *et al.* 2001). However, the relevance of 3′ to 5′ exonuclease activities *in vitro* to the generation of ssDNA by 5′ to 3′ nuclease acitivity *in vivo* at the telomeres of *cdc13-1* mutants is unclear (Booth *et al.* 2001). An alternative model is that the 9-1-1 complex loaded by Rad24 is required to anchor an as yet unidentified 5′ to 3′ exonuclease to DNA (Majka and Burgers 2003).

One nuclease that has the potential to be regulated by *RAD24* in *cdc13-1* mutants is Exo1. Exo1 is involved in the 5′ to 3′ resection of DSBs (Tsubouchi and Ogawa 2000; Tomita *et al.* 2003), in mismatch repair (Szankasi and Smith 1995; Tishkoff *et al.* 1997; Lewis *et al.* 2002), and in meiotic recombination (Khazanehdari and Borts 2000; Kirkpatrick *et al.* 2000). Interestingly, Exo1, like Rad24, contributes to generating ssDNA near the telomeres of *cdc13-1* mutants (Maringele and Lydall 2002). A model showing how checkpoint proteins Ddc1, Mec3, Rad9, Rad17, and Rad24 might regulate Exo1 or other nuclease activities (ExoX) at uncapped telomeres of *cdc13-1* mutants is shown in Figure 1. By carefully characterizing the role of *EXO1*, *RAD9*, and *RAD24*, in regulating ssDNA accumulation and cell cycle arrest of *cdc13-1* mutants, we show that although Exo1 has a critical role in generating ssDNA in *cdc13-1* mutants, *RAD24* appears to regulate a nuclease other than Exo1.

MATERIALS AND METHODS

Yeast strains: All strains used were in the W303 background and unless otherwise indicated contained *RAD5*, rather than the *rad5-535* mutation (Fan *et al.* 1996). Standard genetic procedures of transformation and tetrad analysis were followed (Adams *et al.* 1997). Yeast strains were cultured and serial dilutions tested for growth on plates as previously described (Maringele and Lydall 2002).

Growth at different temperatures: Incubators were set at the temperatures indicated, and in all cases plates at different temperatures were incubated in parallel. The temperatures within incubators oscillated around the set temperature by perhaps 1° or more. Comparatively close temperatures (27.3° and 28.2°) were used because we routinely observe that *cdc13-1 rad9Δ* mutants form colonies less well than *cdc13-1 rad24Δ* mutants do at semipermissive temperatures (see Figure 2R).

Synchronous cultures, viability, cell cycle position, and ssDNA measurements: *bar1 cdc13-1 cdc15-2* strains were released from G1 arrest at 23° and placed at 36°, and cell viability and cell cycle position were monitored as previously described (Lydall and Weinert 1997a). DNA was isolated from cells, and the fraction of ssDNA was measured by quantitative amplification of ssDNA (QAOS) as previously described (Booth *et al.* 2001; Jia *et al.* 2004). In all cases the ssDNA in unknown samples and in standards was measured in triplicate. The primers used to detect ssDNA in the X and Y′ repeats are described in Table 1 and in supplementary material at http://www.genetics.org/supplemental/. The primers used to detect ssDNA at *PDA1*, *YER186C*, and *YER188W* were as previously described (Booth *et al.* 2001; Jia *et al.* 2004).

Microcolony assays: Yeast strains dividing exponentially at 23° were arrested in G1 with α-factor for 2.5 hr. Arrested cells were briefly sonicated, spread as single cells on plates, and incubated for 15 hr at 36° before being photographed at 200× magnification.

RESULTS

***EXO1* contributes to the temperature-sensitive phenotype of *cdc13-1* strains:** To identify nucleases responsible for generating ssDNA near the telomeres of *cdc13-1* mutants we combined mutations in genes encoding known nucleases and other DNA repair proteins with *cdc13-1* and tested the ability of double mutants to grow at a range of temperatures. Removal of gene products that contribute to ssDNA production at telomeres of *cdc13-1* mutants may increase the ability of *cdc13-1* mutants to grow at semipermissive temperatures because lower levels of ssDNA at telomeres should result in less-pronounced cell cycle arrest. In contrast, removal of gene products that inhibit ssDNA production at telomeres of *cdc13-1* mutants should decrease the ability of *cdc13-1* mutants to grow at semipermissive temperatures because higher levels of ssDNA at telomeres should result in greater cell cycle arrest. For example, removal of the DNA repair gene *YKU70* reduces the maximum permissive temperature (MPT) of *cdc13-1* strains (Nugent *et al.* 1998; Polotnianka *et al.* 1998). In contrast, deletion of checkpoint genes like *RAD9* and *RAD24* increases the MPT of *cdc13-1* mutants (Weinert and Hartwell 1993), presumably because checkpoint-defective cells can no longer signal that

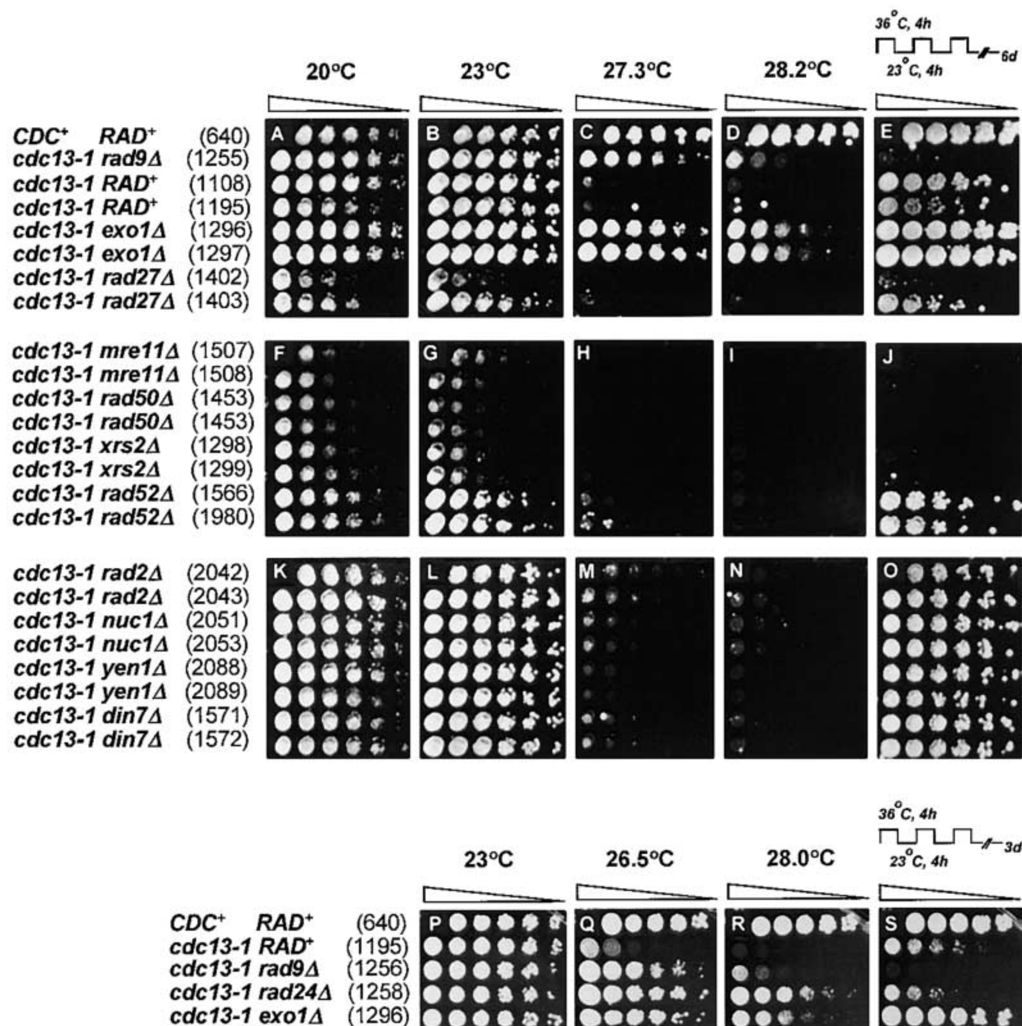

FIGURE 2.—Deletion in *EXO1* permits growth of *cdc13-1* mutants at high temperatures. (A–S) Small aliquots of fivefold dilution series of the strains indicated, and growing at 20°, were transferred to plates and incubated at the temperatures shown for 3 days before being photographed. Some plates (E, J, O, and S) were incubated for three cycles of 36° for 4 hr followed by incubation at 23° for 4 hr and colonies were then allowed to form at 23° for 6 or 3 days. The relevant genotypes are indicated on the left, and strain numbers are shown in parentheses.

ssDNA is present at telomeres and/or because lower levels of ssDNA are present.

We examined the effect of *EXO1* on *cdc13-1* strains since *EXO1* encodes a 5′ to 3′ exonuclease that contributes to, but is not essential for, cell cycle arrest of *cdc13-1* strains (Maringele and Lydall 2002). We also tested the other four nucleases in the Exo1 class encoded by *RAD27* (which is the FLAP endonuclease of budding yeast), *RAD2, DIN7*, and *YEN1* (Fiorentini *et al.* 1997; Johnson *et al.* 1998). In addition we tested Mre11, Rad50, and Xrs2 (components of the MRX complex), which have been shown to function redundantly with Exo1 in generating ssDNA at DSBs in budding yeast (Tsubouchi and Ogawa 2000). Finally, we tested *RAD52*, which is required for virtually all homologous recombination pathways in budding yeast (Paques and Haber 1999), and *NUC1*, which encodes a mitochondrial exonuclease.

Figure 2 shows that *EXO1* is unique among the repair genes tested because, like the *RAD9* checkpoint gene, it inhibited the growth of *cdc13-1* mutants at the semipermissive temperatures of 27.3° and 28.2° (*i.e.*, *cdc13-1 exo1*Δ mutants formed colonies at 27.3°, whereas *cdc13-1 EXO1* strains did not). In contrast, mutations in *MRE11, RAD50, XRS2*, and *RAD27* made *cdc13-1* strains grow poorly, such that even at 20° the double mutants grew slowly as has previously been noted (Nugent *et al.* 1998). These experiments show that the *MRX* complex genes and *RAD27* function to maintain the vitality of *cdc13-1* mutants whereas *EXO1* functions to decrease the vitality of *cdc13-1* mutants. *RAD2, NUC1, YEN1*, and *DIN7* were neutral and did not affect the growth of *cdc13-1* mutants. Grandin *et al.* (2001) have observed that *cdc13-1 mec3*Δ survivor strains that amplify telomeric repeats can grow at higher temperatures. However, this observation is not relevant to the better growth of *cdc13-1 exo1*Δ strains because neither these nor any of the other *cdc13-1* strains we generated at 20° or 23° had amplified telomeric DNA, to generate survivors (see supplementary Figure 1 at http://www.genetics.org/supplemental/).

In comparison to checkpoint-defective *cdc13-1 rad9*Δ cells, *cdc13-1 exo1*Δ mutants were better able to form colonies at 28.2° (Figure 2D) and grew similarly to *cdc13-1 rad24*Δ cells (Figure 2R). Since *rad9*Δ and *rad24*Δ mutants are completely defective in checkpoint-dependent arrest after *cdc13-1*-induced damage, we have assumed that the differences in growth between the *cdc13-1 rad9*Δ

TABLE 1

Primers used to detect ssDNA in telomeric repeats

Primer	Telomeric repeat	Sequence	Type of primer
M 315		AAGGAGCGCAGCGCCTGTA**CCA**	Tag
M 513	X-repeat	AAGGAGCGCAGCGCCTGTA**CCACATTTTAATATCT**	Tagging primer
M 512	X-repeat	*ATTGAGTGGATAGTAGATGGTGAAAAAGTGGTATAACG*	Reverse primer
M 510	X-repeat	TCATTCGGCGGCCCCAAATATTGTATAACTGCCC (underlined)	Probe
M 520		TGCCCTCGCATCGCTCTC**GAA**	Tag
M 521	Y′5000	TGCCCTCGCATCGCTCTC**GAAACAAAGTCAGTGA**	Tagging primer
M 517	Y′5000	*GTCCTGGAACGTTGTCACGAAAAAGC*	Reverse primer
M 516	Y′5000	TGCTAGGCCGAACGACAGCTCTACGATGCGTACTT (underlined)	Probe
M 316		TGCCCTCGCATCGCTCTC**ACA**	Tag
M 243	Y′600	TGCCCTCGCATCGCTCTC**ACAGCCCTATCAG**	Tagging primer
M 237	Y′600	*GAGATCAGCTTGCGCTGGGAGTTACC*	Reverse primer
M 526	Y′600	ACAGGAATGCCGTCCAATGCGGCACTTTAGA (underlined)	Probe

Yeast genome sequences used for primers are formatted differently; tag sequences (not present in the yeast genome) are in regular type, the yeast sequences in tagging primers and tag are in boldface type, reverse primers are in italics, and probes are underlined.

and *cdc13-1 rad24*Δ strains at semipermissive temperatures are due to the more rapid accumulation of single-stranded DNA near the telomeres of *cdc13-1 rad9*Δ mutants (Lydall and Weinert 1995, 1997a). The growth of *cdc13-1 exo1*Δ mutants is consistent with this hypothesis since *EXO1*, like *RAD24*, is important for production of ssDNA near telomeres of *cdc13-1* mutants (Maringele and Lydall 2002).

Interestingly, *cdc13-1 exo1*Δ mutants maintained high viability after three 4-hr cycles at 36° (Figure 2, E and S) and could form large colonies more rapidly than *cdc13-1 EXO1*$^+$ *RAD*$^+$ cells (Figure 2S). This result is consistent with the idea that *EXO1*-dependent ssDNA, accumulating at the telomeres of *cdc13-1* mutants over a 4-hr period at 36°, induces significant growth delay. It is notable that the phenotype of *cdc13-1 rad24*Δ mutants is different from *cdc13-1 exo1*Δ mutants in this assay. They retained reasonable viability, similar to *cdc13-1* cells, but *cdc13-1 rad24*Δ colonies were smaller than *cdc13-1 exo1*Δ colonies after 3 days growth at 23° (Figure 2S).

***EXO1* is required for rapid death of *cdc13-1 rad9*Δ strains:** To test whether Rad9 and Rad24 regulated Exo1, as suggested by Figure 1, we first created combinations of *cdc13-1, exo1*Δ, *rad9*Δ, and *rad24*Δ mutations and measured growth at a range of temperatures (Figure 3). Figure 3B shows that an *exo1*Δ mutation, like a *rad24*Δ mutation, improves the growth of *cdc13-1 rad9*Δ strains at 28.2°, suggesting that Exo1, like Rad24, is required for the accumulation of ssDNA at telomeres in *cdc13-1 rad9*Δ mutants.

If Exo1 contributes to the production of ssDNA in *cdc13-1* mutants, then it may also, like Rad24, contribute to the cell death that occurs when *cdc13-1 rad9*Δ mutants are cultured at 36° for short periods (Lydall and Weinert 1995). To test this, yeast cells growing on plates were subjected to three 4-hr periods at the restrictive temperature of 36°, separated by 4-hr periods of recovery at the permissive temperature 23°. Colonies were then allowed to form at 23°. Figure 3C shows that *cdc13-1 rad9*Δ *exo1*Δ cells formed considerably more colonies than *cdc13-1 rad9*Δ cells did after this protocol. In fact, *cdc13-1 rad9*Δ *exo1*Δ cells formed similar numbers of colonies as *cdc13-1 RAD*$^+$ *EXO1*$^+$ cells and slightly more than *cdc13-1 rad9*Δ *rad24*Δ cells did, with an estimated viability of 20–100%. Figure 3, D–F, shows that *cdc13-1 rad9*Δ *rad24*Δ *exo1*Δ strains behaved similarly to *cdc13-1 rad9*Δ *exo1*Δ strains in this assay.

To confirm that Exo1 has a major role in the cell death that occurs in *cdc13-1 rad9*Δ mutants we measured the ability of *cdc13-1* mutants cultured in liquid at 36° to form colonies when returned to 23°. Figure 3, G and H, confirms that most of the reproductive cell death that occurs in *cdc13-1 rad9*Δ mutants cultured at 36° does not occur if *EXO1* is deleted. Taken together the data in Figures 2 and 3 were consistent with the hypothesis that Exo1 is, like Rad24, responsible for generating ssDNA at the telomeres of *cdc13-1* and *cdc13-1 rad9*Δ mutants and that this ssDNA activates checkpoint control pathways and contributes to cell death.

***exo1*Δ mutants escape from arrest caused by *cdc13-1*-induced DNA damage:** Cells with low levels of ssDNA, or with mutated cell signaling molecules, escape cell cycle arrest more readily than cells with high levels of ssDNA, and this phenomenon has been termed adaptation (Toczyski *et al.* 1997; Lee *et al.* 1998; Vaze *et al.* 2002). In asynchronous cultures *cdc13-1 exo1*Δ mutants arrested cell division less rapidly and completely than *cdc13-1 EXO1*$^+$ cells did (Maringele and Lydall 2002).

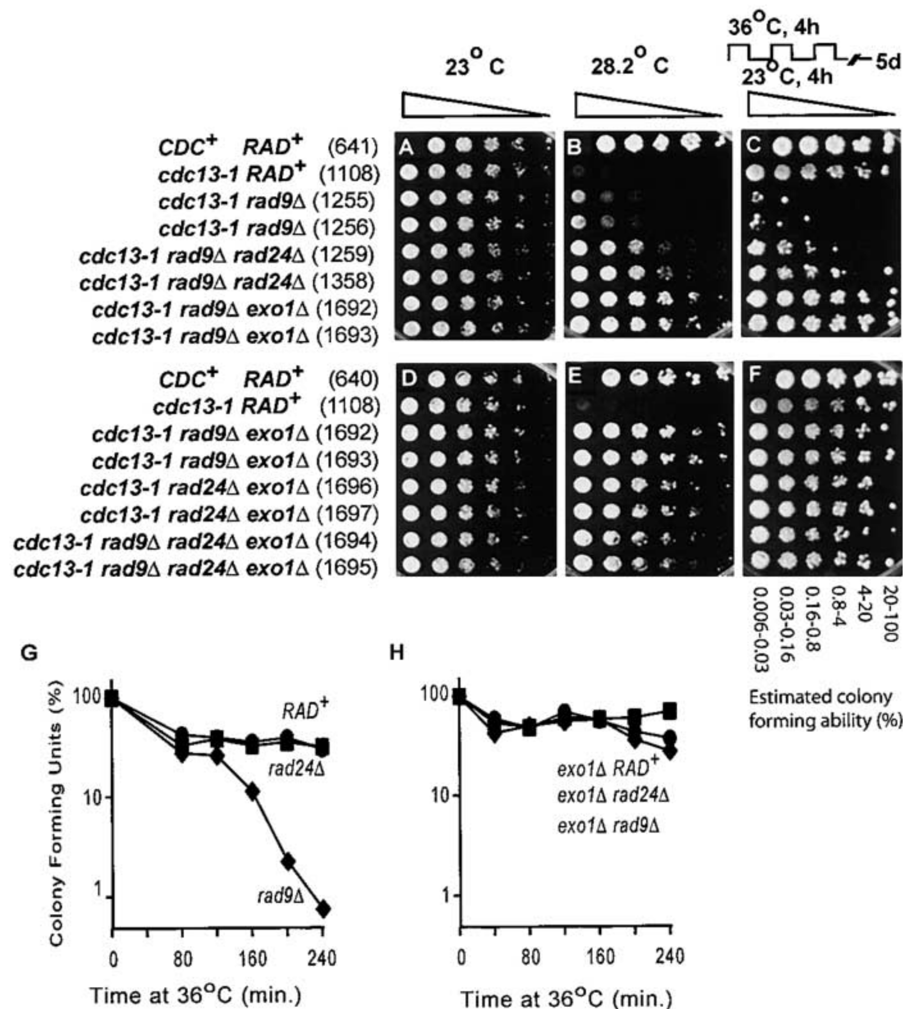

Figure 3.—Exo1 contributes to death of *cdc13-1 rad9*Δ mutants at high temperatures. (A–F) A fivefold dilution series of yeast strains indicated, and growing at 20°C, were transferred to plates and incubated at 23° and 28.2° for 2 days. In addition, some plates (C and F) were incubated for three cycles of 36° for 4 hr followed by incubation at 23° for 4 hr and colonies were then allowed to form at 23° for 5 days. (G) Strains DLY1468 (*RAD*$^+$, squares), DLY1470 (*rad9*Δ, diamonds) and DLY1472 (*rad24*Δ, circles), which carried *bar1 cdc13-1 cdc15-2* and the other mutations specified, were released from G1 arrest to 36°, and the ability of these cells to form colonies was determined. A single, representative experiment is shown. (H) Strains DLY 1431(*exo1*Δ *RAD*$^+$, squares), DLY 1433 (*exo1*Δ *rad9*Δ, diamonds), and DLY 1434 (*exo1*Δ *rad24*Δ, circles) were treated as in G. A single, representative experiment is shown.

To determine whether this impaired cell cycle arrest was due to inefficient arrest, or due to arrest followed by escape from arrest, *bar1* and *cdc15-2* mutations were used to quantify the fraction of *cdc13-1* mutants that had failed to arrest, or escaped arrest, during a single cell cycle (Lydall and Weinert 1997b).

BAR1 encodes a protease that degrades the mating pheromone α-factor. A *bar1* mutation allows efficient G1 arrest of cells with comparatively low levels of α-factor. *CDC15* is required for mitotic exit. At 36° *cdc15-2* mutants arrest cell division in late mitosis with separated chromosomes and an elongated spindle. A population of *bar1 cdc13-1 cdc15-2* mutants arrested in G1 with α-factor at 23° and released from G1 arrest by removing the α-factor and culturing at 36° will go through most of the events of a single cell cycle but not reenter G1. Checkpoint-proficient cells arrest at the metaphase/anaphase checkpoint due to *cdc13-1*-induced damage. Checkpoint-deficient cells do not arrest at metaphase but enter anaphase and arrest at late mitosis due to the *cdc15-2* mutation. The fraction of checkpoint-defective *cdc13-1 cdc15-2* cells that enter anaphase at 36° can be readily measured by examining the position of nuclear DNA within the population (Lydall and Weinert 1997b).

Figure 4A shows that checkpoint-proficient (*RAD*$^+$) *cdc13-1 cdc15-2* cells start to reach medial nuclear division (or metaphase/anaphase) 80 min after release from G1 arrest and that by 120 min >80% of the cells are arrested at medial nuclear division. Arrest at medial nuclear division is efficient since no cells reach late nuclear division (Figure 4B). As expected, checkpoint-defective strains, containing either *rad9*Δ or *rad24*Δ mutations, transiently appeared at medial nuclear division only before entering mitosis (Figure 4A) and accumulated at late nuclear division (the *cdc15-2* arrest point, Figure 4B).

An *exo1*Δ mutation allows a fraction of *cdc13-1* cells arrested at medial nuclear division to escape arrest. Figure 4C shows that *cdc13-1 cdc15-2 exo1*Δ strains were largely arrested at medial nuclear division after 120 min at 36°, like *cdc13-1 RAD*$^+$ strains at 36° (compare Figure 4A with 4C). However, *cdc13-1 cdc15-2 exo1*Δ mutants slowly escaped arrest and accumulated at late nuclear division, such that by 240 min ~30% of *cdc13-1 cdc15-2 exo1*Δ cells had reached late nuclear division (the

cdc15-2 arrest point, Figure 4D). Virtually no *cdc13-1* RAD^+ cells reached late nuclear division in this (Figure 3B) or other experiments (Lydall and Weinert 1995). Arrest of *cdc13-1 cdc15-2 exo1*Δ mutants at medial nuclear division at 36° was completely dependent on *RAD9* and *RAD24* (Figure 4, C and D), indicating that *RAD9*- and *RAD24*-dependent checkpoint pathways are responsible for the initial arrest of *cdc13-1 exo1*Δ mutants. These single cell cycle experiments suggest that an *exo1*Δ mutation allows *cdc13-1* mutants that have arrested cell division to escape arrest.

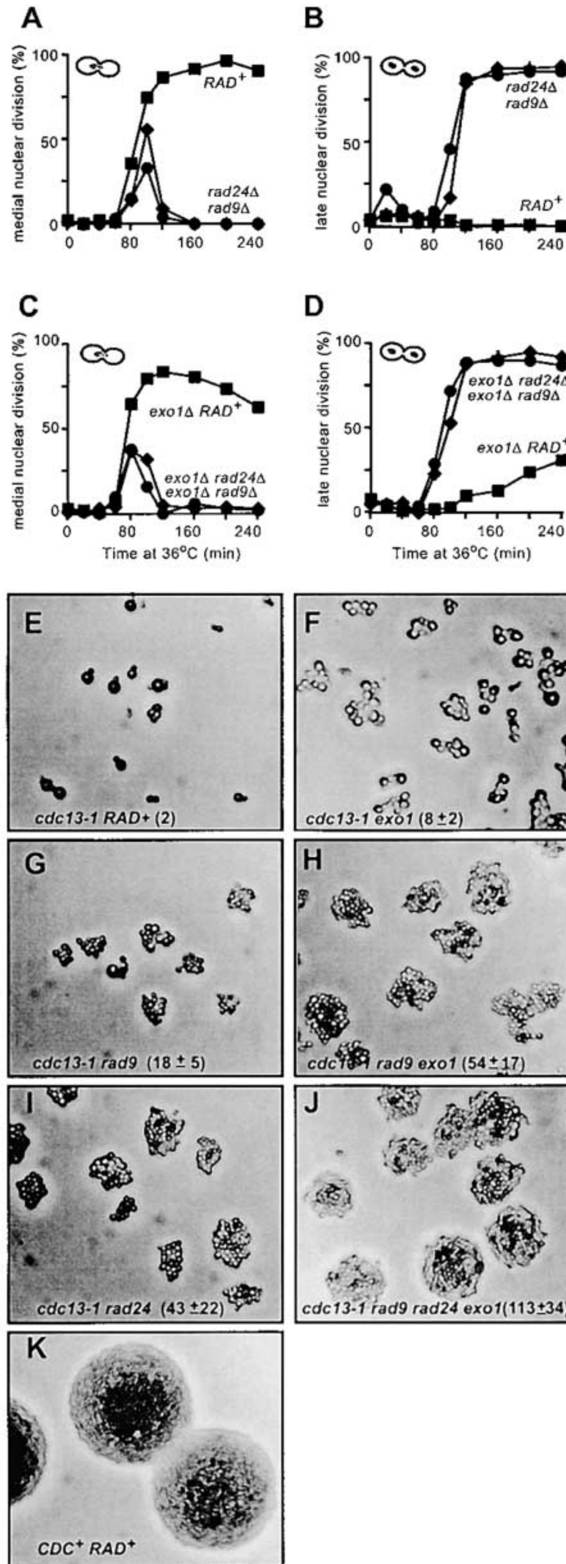

Figure 4.—Deletion in *EXO1* permits escape of *cdc13-1* mutants from arrest at 36°. (A–D) The cell cycle positions of the yeast strains described in Figure 3, G and H, were monitored after staining nuclei with DAPI. A single, representative experiment is shown. (E–K) Yeast strains containing *cdc13-1* (DLY1108), *cdc13-1 exo1*Δ (DLY1296), *cdc13-1 rad9*Δ (DLY1255), and *cdc13-1 rad9*Δ *exo1*Δ (DLY 1692). *cdc13-1 rad24*Δ (DLY1257), *cdc13-1 rad9*Δ *rad24*Δ *exo1*Δ (DLY1695), and CDC^+ (DLY640) were released from G1 arrest and allowed to form microcolonies for 15 hr at 36° before being photographed at 200× magnification. In the W303 genetic background, *cdc13-1* mutants form asymmetric dumbbells after long periods of growth at 36° (E), whereas in other genetic backgrounds the dumbbells remain symmetrical. The cell numbers within microcolonies were estimated from the photographs shown and are indicated, along with standard deviations (E–K). Small microcolonies are largely flat, and all cells are within the focal plane; however, as colony size increases, cells begin to grow out of the focal plane and are not visible.

If an *exo1*Δ mutation allows *cdc13-1* mutants to escape cell cycle arrest and enter anaphase, then *cdc13-1 exo1*Δ mutants might be able to complete cell division and to divide. If so then after long periods of growth at 36° *cdc13-1 exo1* mutants should form larger microcolonies than *cdc13-1* strains do. To test this, we arrested single *MAT***a** *cdc13-1* cells in G1 using the mating pheromone α-factor and incubated them on plates for 15 hr at 36°. Figure 4, E–J, shows the effect of *exo1*Δ, *rad9*Δ, and *rad24*Δ mutations on the ability of *cdc13-1* strains to divide and form microcolonies at 36°. It is clear that an *exo1*Δ mutation increased the size of *cdc13-1* microcolonies. Figure 4E shows that *cdc13-1* cells mainly arrested cell division with two buds when cultured at 36°. In contrast, *cdc13-1 exo1*Δ mutants formed larger microcolonies, with most of the single cells eventually forming colonies of 5–10 cells after 15 hr at 36° (Figure 4F).

It is notable that most individual *cdc13-1 exo1*Δ cells were larger than the checkpoint defective *cdc13-1 rad9*Δ cells in microcolonies grown under identical conditions (compare Figure 4F with 4G). This observation is consistent with the existence of a checkpoint that extends each cell cycle of *cdc13-1 exo1*Δ mutants (see Figure 4, C and D), and that while arrested at this checkpoint *cdc13-1 exo1*Δ cells enlarge in size before escaping arrest.

Exo1 inhibits the growth of *cdc13-1 rad9*Δ colonies: We have previously shown that *cdc13-1 rad24*Δ and *cdc13-1 rad9*Δ *rad24*Δ mutants form larger microcolonies than *cdc13-1 rad9*Δ cells do at 36° (Lydall and Weinert 1997a). Figure 4, H and I, shows that Exo1, like Rad24, appears to inhibit the division of *cdc13-1 rad9*Δ cells since *cdc13-1 rad9*Δ *exo1*Δ triple mutants form large-sized colonies, like *cdc13-1 rad24*Δ cells. This is consistent with

the hypothesis that *RAD24*- and *EXO1*-dependent ssDNA production limits the division of *cdc13-1 rad9*Δ cells at 36°.

If Rad24 and Exo1 contribute to the same pathway to limit the division of *cdc13-1 rad9*Δ cells at 36°, then *cdc13-1 rad9*Δ *rad24*Δ *exo1*Δ quadruple mutants should form colonies similar in size to those of *cdc13-1 rad9 rad24*Δ or *cdc13-1 rad9*Δ *exo1*Δ triple mutants. This logic explains why a *cdc13-1 rad9*Δ *rad17*Δ *rad24*Δ *mec3*Δ mutant forms microcolonies similar in size to those of *cdc13-1 rad9*Δ *rad17*Δ and other similar triple mutants (Lydall and Weinert 1997a). However, if Rad24 and Exo1 contribute to independent pathways to limit division, then *cdc13-1 rad9*Δ *rad24*Δ *exo1*Δ quadruple mutants may form larger colonies than the corresponding triple mutants do. Figure 4J shows that *cdc13-1 rad9*Δ *rad24*Δ *exo1*Δ mutants do indeed form larger colonies than the corresponding triple mutants do. This suggests that Rad24 and Exo1 play different roles in limiting the division *of cdc13-1 rad9*Δ cells at 36° and is consistent with different growth of *cdc13-1 exo1*Δ *vs. cdc13-1 rad24*Δ mutants under other conditions, *e.g.*, Figure 2S.

Msh2 does not contribute to cell cycle arrest of *cdc13-1* mutants: Exo1 binds Msh2, a core component of eukaryotic mismatch repair pathways, and plays an important role in mismatch repair (Szankasi and Smith 1995; Tishkoff *et al.* 1997; Marti *et al.* 2002). To test whether Msh2, like Exo1, regulated cellular responses to the *cdc13-1* defect, we examined the effect of deleting *MSH2* (Luhr *et al.* 1998). Although some early colony growth experiments suggested that Msh2 played a role in responding to the *cdc13-1* defect, we concluded after more experiments that Msh2 plays no direct role in recruiting Exo1 to *cdc13-1*-defective telomeres (see Supplementary Figure 2 at http://www.genetics.org/supplemental/).

Exo1 is required for production of ssDNA at X repeats and single-copy subtelomeric sequences in *cdc13-1* mutant cells: To assess directly the role of Exo1 in generating ssDNA in *cdc13-1* mutants we used synchronous cultures to examine ssDNA production at three repetitive loci found on numerous telomeres and three single-copy loci near the right telomere of chromosome V. Previously we showed that in *cdc13-1* mutants ssDNA is generated in a telomere-to-centromere direction, with Rad9 inhibiting ssDNA production, and Rad24 being required for ssDNA production, and that ssDNA exists at least 30 kb from the telomere in *cdc13-1 rad9* mutants (Lydall and Weinert 1995; Booth *et al.* 2001; Jia *et al.* 2004). We have also demonstrated that ssDNA accumulation depends on release from G1 (M. K. Zubko and D. Lydall, unpublished data).

Figure 5A indicates the two major telomere types found in budding yeast. All telomeres contain X repeats. Approximately half of the telomeres possess X repeats directly adjacent to the TG repeats within 1 kb of the chromosome terminus and are termed X-type telomeres. The other class of telomeres contains one or more Y′ repeats between the X and TG repeats, which are termed Y-type telomeres (Pryde *et al.* 1997). Subtelomeric Y′ repeats are highly dynamic, and their location and number vary between yeast strains (Louis *et al.* 1994). According to the Saccharomyces Genome Database, chromosome V of the sequenced S288C strain contains a single Y′; single X; and the single-copy genes *YER188W*, *YER186C*, and *PDA1* at the indicated distances from the chromosome end. The W303 strains used in this study are reasonably closely related to S288C strains (Winzeler *et al.* 2003), but it is possible that there are different types or numbers of subtelomeric repeats, at this chromosome end, in W303 strains, and also that differences have arisen between strains while undergoing genetic crosses (Horowitz *et al.* 1984). For these reasons the distances of the loci from the end of chromosome V should be considered approximate and are shown in parentheses.

The accumulation of ssDNA in *cdc13-1 EXO1*$^+$ and *cdc13-1 exo1*Δ mutants was measured by quantitative amplification of ssDNA (QAOS; Figure 5, B–G). In QAOS a tagging primer anneals to ssDNA but not to dsDNA at low temperature, and then primer extension creates a complementary, tagged, ssDNA-dependent molecule, which is detected by quantitative real-time PCR (Booth *et al.* 2001). QAOS can accurately measure ssDNA in single-copy yeast genes at levels $>0.2\%$. Figure 5B shows that neither *cdc13-1* nor *cdc13-1 exo1*Δ mutants generated significant levels of ssDNA at the *PDA1* locus, 30 kb from the VR telomere, consistent with earlier experiments (Booth *et al.* 2001; Jia *et al.* 2004). However, closer to the telomere, at the *YER186C* locus (14,500 bp from the telomere), it is clear that *cdc13-1 EXO1*$^+$ cells generated significant levels of ssDNA while *cdc13-1 exo1*Δ strains did not (Figure 5C). We rarely observe ssDNA at single-copy sequences rising much above 10% and have been unable to determine whether this is due to degradation of ssDNA in *cdc13-1* mutants *in vivo* or during DNA preparation or because telomeres are only partially susceptible to nuclease activity *in vivo*; see discussion in Booth *et al.* (2001). At *YER186C cdc13-1 EXO1*$^+$ strains began to accumulate ssDNA 120 min after releasing a G1 culture to 36° and reached a level of 5–6% ssDNA by 240 min, whereas *cdc13-1 exo1*Δ strains did not generate ssDNA above 1%. Closer to the telomere, at *YER188W* and in the repetitive X sequence, a similar pattern to that at *YER186C* was seen, with very little ssDNA being observed in *cdc13-1exo1*Δ cells, but significant levels being observed in *cdc13-1 EXO1*$^+$ cells (Figure 5, D and E). These data demonstrate that Exo1 is essential for generating the vast majority of ssDNA at X repeats and single-copy sequences at telomeres of *cdc13-1* mutants at 36°.

At repetitive Y′ repeats, found on approximately half of the telomeres, ssDNA levels increased significantly in *cdc13-1exo1*Δ cells (Figure 5, F and G; Maringele and Lydall 2002). We measured ssDNA ∼600 and 5000 bp from the telomeric ends of the Y′ repeats. Approxi-

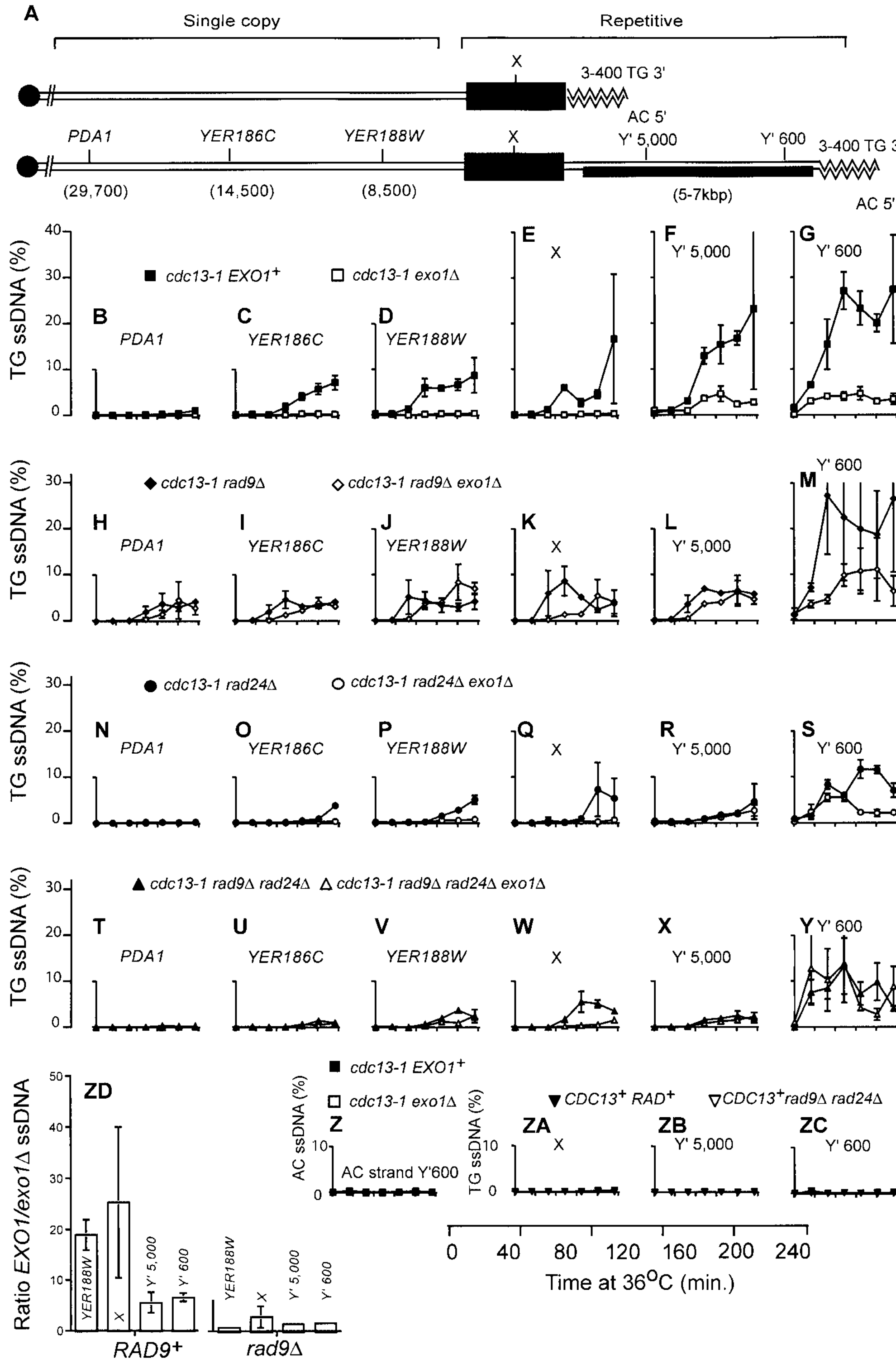

Figure 5.—Exo1 is required to generate ssDNA in X and single-copy telomeric sequences in *cdc13-1* mutants. (A) A schematic model of the two classes of telomere in budding yeast. One class contains an X repeat, but no Y′ repeats, and the other class contains one or more Y′ repeats, in addition to the X repeats. The bottom half of A is a representation of the right telomere of the sequenced chromosome V present in the Saccharomyces Genome Database. It comprises a 3- to 400-bp TG/AC repeat, a Y′ repeat, a 374-bp X repeat, and the *YER188W, YER186C,* and *PDA1* single-copy loci. Using primers and probes directed to repetitive and single-copy loci we were able to detect the appearance of ssDNA in repetitive elements (at numerous telomeres, including 5R) and also specifically 8500, 14,500 and 29,700 bp from the right telomere of chromosome V. Yeast strains were released from G1 arrest to 36°, and the amount of ssDNA was measured by quantitative amplification of ssDNA (QAOS; Booth *et al.* 2001). In most cases the data points indicate the average amount of ssDNA measured in two independent strains of identical genotype, with error bars indicating the difference observed between the two strains. When the amount of ssDNA in a genotype had been previously measured (Booth *et al.* 2001), a single new experiment was performed with error bars representing the standard error of the mean of three independent measurements. In cases where a single strain of a particular genotype was identified, two independent synchronous cultures of that strain were performed and the difference in values between the two experiments is indicated by the error bars. (B–G) Yeast stains containing *cdc13-1* (DLY1468 and DLY1469, solid squares) and *cdc13-1 exo1*Δ (DLY1431 and DLY1432, open squares) mutations. (H–M) Yeast strains containing *cdc13-1* rad9Δ (DLY1470 and DLY1471, solid diamonds) and *cdc13-1* rad9Δ *exo1*Δ (DLY1433 and DLY1476, open diamonds) mutations. (N–S) Yeast strains containing *cdc13-1 rad24*Δ (DLY1472, single experiment, solid circles) and *cdc13-1* rad24Δ *exo1*Δ (DLY1434, duplicate experiments, open circles) mutations. (T–Y) Yeast strains containing *cdc13-1 rad9 rad24*Δ (DLY1474, single experiment, solid triangles) and *cdc13-1rad9*Δ *rad24*Δ *exo1*Δ (DLY1435 and 1477, open triangles) mutations. (Z) Yeast strains and symbols are as in G, and ssDNA was measured on the AC strand. (ZA–ZC) Yeast strains containing *CDC13+ cdc15-2 RAD*+ (DLY 1363, single experiment, solid downward-pointing triangles) and *CDC13*+ *rad9*Δ *rad24*Δ *cdc15-2* (DLY1414, single experiment, open downward-pointing triangle) mutations. (ZD) A histogram showing the ratio between the amount of ssDNA observed at four telomeric loci in *cdc13-1 EXO1*+ *vs. cdc13-1 exo1*Δ strains and corresponding *rad9*Δ strains. Ratios shown are average ratios of ssDNA in *EXO1*+ *vs. exo1*Δ strains at 120-, 160-, 200-, and 240-min time points. The ratios were determined from the data plotted in D–G and J–M. The error bars show the standard error of the mean.

mately 600 bp from the end of the telomere, ssDNA reached ∼5% 40 min after releasing G1-arrested strains to 36° and remained close to this level for the remaining 200 min. These levels were lower than those seen in *cdc13-1 EXO1*$^+$ cells, which reached 20–30%. At the Y′5000 locus ssDNA levels were similar to those at Y′600, but the kinetics of appearance were slower, with ssDNA not accumulating beyond 1% until 80–120 min after release from G1 arrest (Figure 5F). This suggests that a 5′ to 3′ nuclease degrades the telomere beginning at the telomeric end.

If ssDNA in *cdc13-1* mutants initiates at the chromosome terminus and extends toward the centromere, as suggested by the data here (Figure 5, B–G) and obtained earlier (Booth *et al.* 2001), then the termini of X-type telomeres appear to have different properties to the termini of Y′-type telomeres. At X-type telomeres, which contain no Y′ repeats and represent approximately half the telomeres in budding yeast, the X repeats lie within 1 kb of the chromosome end at a similar position to the Y′600 locus of Y′-type telomeres (Figure 5A). Yet, on average, the amount of ssDNA observed at X repeats in *cdc13-1 exo1*Δ mutants is considerably less than even half the amount of ssDNA observed at the Y′600 or Y′5000 loci. The left part of Figure 5ZD shows the ratio of ssDNA observed in *cdc13-1 EXO1 vs. cdc13-1 exo1*Δ mutants at *YER188W*, X, Y′5000 and Y′600 repeats. At *YER188W* and X repeats *EXO1*$^+$ cells contain ∼20-fold more ssDNA that *exo1*Δ mutants do. However, in the Y′ repeats the differential is reduced to ∼6-fold. Thus, Exo1 is more important for generating ssDNA at X and single-copy telomeric sequences than in Y′ repeats of *cdc13-1* mutants.

In summary, the data in Figure 5, B–G, are consistent with ssDNA in *cdc13-1* mutants being generated by two, or more, 5′ to 3′ exonucleases. Exo1 is critical for the production of ssDNA in X repeats and of single-copy sequences on the right telomere of chromosome V (Maringele and Lydall 2002). A significant amount of ssDNA in Y′ repeats is also dependent on *EXO1* but, in addition, an *EXO1*-independent nuclease(s) appears able to generate ssDNA in the repetitive Y′ sequences.

Rad9 inhibits Exo1 and other nucleases: Most of the ssDNA and cell death that occur in *cdc13-1 rad9*Δ mutants are dependent on Rad24 (Lydall and Weinert 1995). Since an *exo1*Δ mutation rescues the rapid loss of viability observed in *cdc13-1 rad9*Δ mutants (Figure 3) it seemed likely that Exo1 would be required, like Rad24, for rapid generation of ssDNA in *cdc13-1 rad9*Δ mutants. To test this directly, we examined ssDNA production in *cdc13-1 rad9*Δ and *cdc13-1 exo1*Δ *rad9*Δ strains (Figure 5, H–M). Surprisingly, the effect of deleting *EXO1* on ssDNA production in *cdc13-1 rad9*Δ mutants was considerably less than the effect of deleting *RAD24*. *cdc13-1 rad9*Δ *exo1*Δ mutants clearly generated significant levels of ssDNA at all telomeric loci tested (Figure 5, H–M), whereas a *cdc13-1 rad9*Δ *rad24*Δ strain generated considerably less ssDNA, particularly at loci further from the telomere (Figure 5, T–Y).

At all loci examined the accumulation of ssDNA is marginally slower in *cdc13-1 exo1*Δ *rad9*Δ strains than in *cdc13-1 rad9*Δ strains. At *PDA1*, a locus that becomes significantly single stranded only in *cdc13-1 rad9*Δ mutants but not *in cdc13-1 RAD*$^+$ mutants, *cdc13-1 exo1*Δ *rad9*Δ mutants clearly generate significant levels of ssDNA reaching ∼5% (Figure 5, B and H). In *cdc13-1 rad9*Δ *exo1*Δ mutants the kinetics of ssDNA accumulation appear to be ∼40–80 min delayed in comparison with *cdc13-1 rad9*Δ *EXO1*$^+$ cells. This is apparent at *PDA1, YER186C, YER188W,* the X, and the Y′5000 loci, where the ssDNA reaches a level >1% ∼40 min later (Figure 5, H–L). Therefore, it appears that Rad9 inhibits *EXO1*-dependent nuclease activity to some extent.

Comparison between Figure 5, B–E, and 5, H–K, demonstrates that while Exo1 is critical for generation of ssDNA in the X sequences and the single-copy sequences that lie internal to these in *cdc13-1*strains (Figure 5, B–E), Exo1 is much less important in this process in *cdc13-1 rad9*Δ strains (Figure 5, H–K). Figure 5ZD illustrates this because it shows the *EXO1* independence of ssDNA production in *cdc13-1 rad9*Δ cells at both repetitive and single-copy sequences (right part of the figure) compared with the corresponding *RAD9*$^+$ cells. The ssDNA that appears in *cdc13-1 rad9*Δ *exo1*Δ strains is clearly Exo1 independent, and it might be generated by a different nuclease, one that is normally inhibited by Rad9. Furthermore, these data suggest that Rad9 contributes to the integrity of some type of barrier or domain structure in *cdc13-1* strains that ensures that ssDNA generation in X and single-copy telomeric sequences is largely dependent on Exo1.

Exo1 and Rad24 control nucleases with different properties: Exo1 and Rad24 are each required for the efficient generation of ssDNA in *cdc13-1* mutants (Figure 5; Lydall and Weinert 1995; Booth *et al.* 2001). A simple model to explain these data is that the Rad24, RFC-like protein (Lydall and Weinert 1997a; Green *et al.* 2000) is required to load or in some other manner to regulate the activity of Exo1. If so, then *cdc13-1 exo1*Δ *rad24*Δ triple mutants should behave like *cdc13-1 exo1*Δ and *cdc13-1 rad24*Δ double mutants. Figure 5, N–S, shows that the patterns of ssDNA accumulation in *cdc13-1 rad24*Δ and *cdc13-1 rad24*Δ *exo1*Δ mutants are different. *cdc13-1 rad24*Δ *exo1*Δ mutants behave like *cdc13-1 exo1*Δ mutants and generate very little ssDNA in the X repeat and the single-copy sequences that lie internal to these. In contrast, *cdc13-1 rad24*Δ strains generate small but significant amounts of ssDNA at *YER186C, YER188W,* and the X repeat at late time points (Figure 5, O–Q). One explanation for these data is that Exo1 is essential for ssDNA production in the X and single-copy sequences and that Rad24 is only partially required for the activity of Exo1. However, examination of ssDNA accumulation in *cdc-13-1 rad9*Δ mutants suggests that this simple explana-

tion is insufficient. Rad24 is required for most of the ssDNA produced in *cdc13-1 rad9*Δ mutants (Figure 5, T–Y; Lydall and Weinert 1995; Booth *et al.* 2001) whereas Exo1 is not (Figure 5, H–M). This suggests that Rad9 plays a major role in inhibiting a *RAD24*-dependent, but *EXO1*-independent, nuclease that generates ssDNA in *cdc13-1* mutants.

Exo1- and Rad24-independent nuclease activity in *cdc13-1* mutants: To determine if Exo1 is responsible for the small amount of ssDNA that accumulates near telomeres of *cdc13-1 rad9*Δ *rad24*Δ mutants, we examined the ssDNA accumulation in *cdc13-1 rad9*Δ *rad24*Δ *exo1*Δ quadruple mutants (Figure 5, T–Y). We found significant levels of ssDNA appearing in the Y′ sequences of *cdc13-1 rad9*Δ *rad24*Δ *exo1*Δ mutants (Figure 5Y). However, in the X sequences and those internal to the X sequences most but not all of the ssDNA that formed in *cdc13-1 rad9*Δ *rad24*Δ mutants was dependent on Exo1 (Figure 5, T–W). The observation that *cdc13-1 rad24*Δ *exo1*Δ strains generate significant levels of ssDNA at the Y′600 locus (Figure 5S) also demonstrates that Exo1- and Rad24-independent mechanisms must exist to generate ssDNA in the Y′ sequences of *cdc13-1* mutants.

Finally, experimental controls show that no detectable ssDNA accumulates on the strand that ends with the 5′ AC repeats at the telomere, in either *cdc13-1* or *cdc13-1 exo1*Δ mutants (Figure 5Z), and this is consistent with earlier studies on *cdc13-1* mutants (Garvik *et al.* 1995). Figure 5, ZA–ZC, shows that all the ssDNA generated in the Y′ and X sequences is dependent on the *cdc13-1* defect.

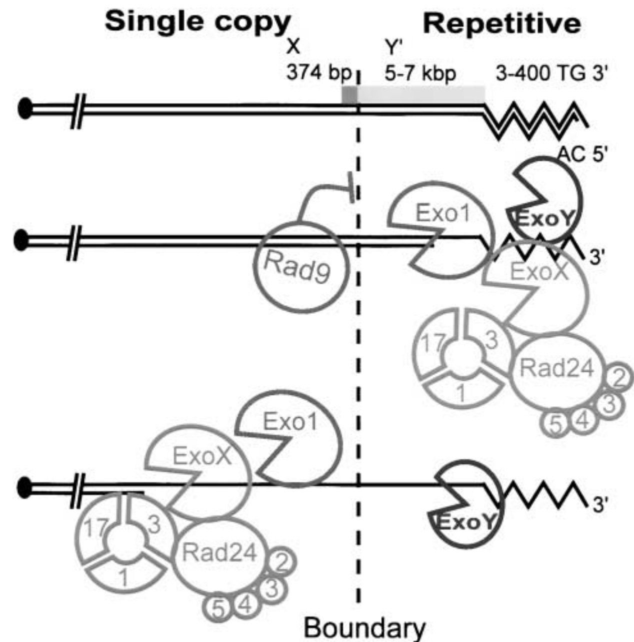

Figure 6.—A model for the interaction between nucleases and checkpoint proteins at *cdc13-1*-induced damage. According to this model, ssDNA formation begins at the chromosome end (where Cdc13p binds). Exo1, ExoX (which is Rad17, Rad24, Mec3, and Ddc1 dependent), and ExoY contribute to ssDNA production. Rad9 inhibits exonuclease activity by contributing to a nuclease progression barrier centered on X repeats. Exo1 and ExoX are both critical for generating ssDNA beyond the Rad9-dependent barrier. However, if the barrier is missing, due to the absence of Rad9, then ExoX becomes more important than Exo1 for generating ssDNA at single-copy sequences near telomeres. ExoY generates ssDNA in the absence of Exo1 and ExoX.

DISCUSSION

Telomeres contain various types of repetitive DNA structures and a large number of telomere-binding proteins that function to protect the telomere from repair and checkpoint pathways (Blackburn 2001; Cervantes and Lundblad 2002; Lydall 2003; Ferreira *et al.* 2004; Harrington 2004). In this article we have begun to dissect the interactions that occur between Rad9 and Rad24 checkpoint products and the Exo1 DNA repair protein at unprotected telomeres of budding yeast *cdc13-1* mutants.

We establish that Exo1 is unique among products of 10 different DNA repair genes tested because like the Rad9 and Rad24 checkpoint proteins, it inhibits the growth of *cdc13-1* mutants at semipermissive temperatures of ∼27°. In contrast, components of the MRX complex, Mre11, Rad50, and Xrs2, along with the FLAP endonuclease Rad27, have opposite properties to Exo1, and they contribute to the vitality of *cdc13-1* strains at the permissive temperature of 23°. Other nucleases and DNA repair proteins encoded by *RAD52, RAD2, MSH2, NUC1, YEN1*, and *DIN7* played no detectable role at the telomeres of *cdc13-1* mutants because they neither inhibit nor enhance growth of *cdc13-1* mutants at 23°.

There is evidence for overlapping functions between the MRX complex and Exo1 in DNA repair (Tsubouchi and Ogawa 2000; Moreau *et al.* 2001; Lee *et al.* 2002; Lewis *et al.* 2002). Indeed, the MRX complex functions as a nuclease to create ssDNA at telomeres created *de novo* (Diede and Gottschling 2001). It is possible that MRX plays a role in generating ssDNA in *cdc13-1* mutants and represents ExoX or ExoY in Figure 6, but we have been unable to test this directly because *cdc13-1 mrx*Δ double mutants grow extremely poorly even at 20° (Figure 2). It is likely that the protective role of MRX at telomeres (Nugent *et al.* 1998; Maringele and Lydall 2002), or its role in recruiting telomerase (Tsukamoto *et al.* 2001), explains the poor growth of *cdc13-1 mrx*Δ double mutants. It is clear that Exo1 has very different properties to the components of the MRX in the context of the *cdc13-1*- and *yku70*Δ-induced telomere damage complex (this work and Nugent *et al.* 1998; Maringele and Lydall 2002).

Exo1 is a mismatch repair-associated exonuclease, and some studies in mammalian cells suggest that mismatch repair pathways contribute to DNA damage checkpoint pathways (Davis *et al.* 1998; Yan *et al.* 2001). In-

TABLE 2

Nuclease activities at unprotected telomeres

	Important for telomeric ssDNA production in			
	Single-copy sequences in *cdc13-1* cells	Single-copy sequences in *cdc13-1 rad9*Δ cells	Y′ repeats in *cdc13-1* cells	Y′ repeats in *yku70*Δ cells
Exo1	Yes	No	Partially	Yes
ExoX (Rad24-dependent)	Yes	Yes	Partially	No
ExoY	?	?		?

deed, recent experiments show that human Msh2 binds to human checkpoint PI3 type kinase, ATR (orthologue of budding yeast Mec1; Wang and Qin 2003). However, other studies have questioned the role of mismatch repair in checkpoint control (Aquilina *et al.* 1999; Strathdee *et al.* 2001). Furthermore, mismatch repair pathways regulate the growth of cells growing without telomerase (Rizki and Lundblad 2001). Our analyses lead us to conclude that Msh2, a core component of the mismatch repair machinery, plays no essential role in either recruiting either Exo1 or other nucleases or signaling cell cycle arrest, in *cdc13-1* mutants.

Analysis of ssDNA production in *cdc13-1* yeast strains containing combinations of *exo1*Δ *rad9*Δ and *rad24*Δ mutations shows that regulation of ssDNA production by nucleases and checkpoint pathways is complex. Our data support a model in which at least three independent nucleases attack the telomeres of *cdc13-1* mutants at 36° (Figure 6). Exo1 is the primary nuclease active at the telomeres of *cdc13-1* mutants (this work) and at telomeres of *yku70*Δ mutants at 37° (Maringele and Lydall 2002). ExoX and ExoY are as yet unidentified and play a lesser role in generating ssDNA. Their properties are described in Table 2 and below.

We consider Exo1 the primary nuclease for generating ssDNA in *cdc13-1* mutants because Exo1 is critical for generating ssDNA in X repeats and single-copy sequences internal to X. In addition, Exo1 is important for generating high levels of ssDNA in the Y′ repeats of *cdc13-1* mutants. However, when Rad9 is missing, other nucleases, in particular a Rad24-dependent nuclease, designated ExoX, can generate ssDNA in single-copy sequences of *cdc13-1* mutants. ExoX can be proposed because *cdc13-1 rad9*Δ *rad24*Δ strains (deficient in ExoX, due to the absence of Rad24, but proficient in Exo1) generate very little ssDNA internal to the X repeats, whereas *cdc13-1 rad9*Δ *exo1*Δ strains (deficient in Exo1 but proficient in ExoX) are able to generate high levels of ssDNA at these loci. The putative Rad24-dependent ExoX is, like Exo1, important for generating maximum levels of ssDNA in the Y′ repeats of *cdc13-1* mutants. ExoY is another putative nuclease that generates ssDNA near the telomeres of *cdc13-1 exo1*Δ *rad24*Δ mutants. Alternatively, ExoY could be the same nuclease as ExoX but with an activity partially dependent on Rad24.

ExoX is not yet identified. ExoX may be the intrinsic nuclease activity of the checkpoint sliding clamp, Rad17, Mec3, and Ddc1 or may be another, so far unidentified 5′ to 3′ nuclease tethered to DNA by this sliding clamp. Alternatively, ExoX and/or ExoY may be some other combination of repair activities, *e.g.*, combined helicase and endonuclease activities, or MRX activity. Further experiments will be necessary to define ExoX.

Our experiments show that Exo1 is critical for generating ssDNA at X repeats and single-copy subtelomeric sequences when Rad9 is present in *cdc13-1* mutants, but Exo1 is less critical in Y′ repeats or in X repeats when Rad9 is missing (Table 2). Interestingly, Pryde and Louis have shown that there is a domain of transcriptional repression centered on the X repeat at telomeres; *i.e.*, Y′ repeats are less transcriptionally silenced than X repeats (Pryde and Louis 1999). It seems plausible that this domain of transcriptional repression might share properties with a nuclease inhibition domain since it is located in a similar position. Other experiments suggest that Rad9 inhibits nuclease activity in *cdc13-1* mutants by both kinase-dependent (Rad53 and Mec1) and kinase-independent mechanisms (Jia *et al.* 2004). Further experiments will be required to elucidate how Rad9 inhibits nucleases at uncapped telomeres.

We began our studies with the assumption that *cdc13-1 rad9*Δ mutants became rapidly inviable at 36° because of the rapid accumulation of ssDNA. *exo1*Δ and *rad24*Δ mutations each suppress the rapid loss of viabilty observed in *cdc13-1 rad9*Δ mutants, but *cdc13-1 rad9*Δ *exo1*Δ mutants, in contrast to *cdc13-1 rad9*Δ *rad24*Δ mutants, still generate high levels of ssDNA. This puzzle may be explained if Exo1 contributes directly to the loss of viability of *cdc13-1 rad9*Δ cells through enzymatic activities other than its 5′ to 3′ exonuclease activity. For example, Exo1 possesses FLAP endonuclease activity (Lee and Wilson 1999; Tran *et al.* 2002), and this activity could be responsible for forming cytotoxic lesions in *cdc13-1 rad9*Δ mutants. Other recent experiments show that Mec1 and Rad53 also contribute to the loss of viability of *cdc13-1 rad9*Δ strains and yet, like Exo1, they do not greatly affect the rate of accumulation of ssDNA (Jia *et al.* 2004).

Finally, analysis of cell cycle arrest in *cdc13-1 exo1*Δ mutants allows us to address the role of telomeric ssDNA

in cell cycle arrest. Our data suggest that *cdc13-1exo1*Δ strains generate ssDNA in Y′ repeats, but not internally to these. After 4 hr incubation ∼30% of *cdc13-1exo1*Δ strains escape arrest without apparently removing or "repairing" the ssDNA (Figure 5, F and G). We assume, but have no direct evidence, that these *cdc13-1* cells dividing in the presence of ssDNA at telomeres have downregulated checkpoint signal transduction pathways, as has been described at double-strand breaks (Leroy *et al.* 2003). The amount of ssDNA present in *cdc13-1 exo1*Δ strains can be estimated at ∼15 kb, on the basis that there are ∼40 Y′ repeats in G2 cells (64 telomeres), each with an average size of 6 kb, and 64 telomeric TG repeats with an average size of 350 bp, and 5% (13 kb) of this 260-kb sequence is single stranded. This value is of a similar order to the amount of ssDNA required to arrest cell division in cells with a single unrepaired DSB (between 4.6 and 25 kb; Vaze *et al.* 2002) or with stalled replication forks (Sogo *et al.* 2002). This comparison argues that exposed telomeric ssDNA is as efficient as ssDNA generated at DSBs elsewhere in the genome in activating checkpoint-dependent arrest.

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