Single-Stranded DNA Arising at Telomeres in *cdc13* Mutants May Constitute a Specific Signal for the *RAD9* Checkpoint

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A *cdc13* temperature-sensitive mutant of *Saccharomyces cerevisiae* arrests in the G₂ phase of the cell cycle at the restrictive temperature as a result of DNA damage that activates the *RAD9* checkpoint. The DNA lesions present after a failure of Cdc13p function appear to be located almost exclusively in telomere-proximal regions, on the basis of the profile of induced mitotic recombination. *cdc13 rad9* cells dividing at the restrictive temperature contain single-stranded DNA corresponding to telomeric and telomere-proximal DNA sequences and eventually lose telomere-associated sequences. These results suggest that the *CDC13* product functions in telomere metabolism, either in the replication of telomeric DNA or in protecting telomeres from the double-strand break repair system. Moreover, since *cdc13 rad9* cells divide at a wild-type rate for several divisions at the restrictive temperature while *cdc13 RAD9* cells arrest in G₂, these results also suggest that single-stranded DNA may be a specific signal for the *RAD9* checkpoint.

Many temperature-sensitive mutations that cause cells to arrest at specific stages in the cell cycle have been isolated in the yeast *Saccharomyces cerevisiae* (49). Mutations in four genes, *CDC2*, *-9*, *-13*, and *-17*, result in arrest in late S or G₂ phase. Cells defective for these genes probably arrest because of defects in the completion of DNA replication. *CDC2*, *-9*, and *-17* are all known to encode components of the DNA replication machinery: *CDC2* and *CDC17* code for DNA polymerases (5, 13), whereas *CDC9* codes for DNA ligase (36). Furthermore, mutations in all four genes induce high levels of mitotic recombination at the restrictive temperature, a result consistent with the presence of DNA lesions that remain after incomplete DNA replication (25). *RAD9* is part of a system of genes that detects the presence of X-ray-induced DNA damage and stops the cell cycle in G₂ to allow time for repair before the cell enters mitosis (64). The *RAD9* checkpoint may also recognize spontaneous damage and/or incomplete DNA replication, since *rad9* deletion mutants experience an elevated level of chromosome loss (65). In a *rad9* mutant background, cells with mutations in any one of these four *CDC* genes fail to arrest in G₂; instead they proceed into the next cell cycle. This result has been interpreted to mean that mutations in all four genes leave damage after DNA replication and that this damage produces a G₂ arrest mediated by the *RAD9* pathway (66). In a *RAD9* background, these mutants retain viability for a few hours at the restrictive temperature because mitosis in the presence of DNA damage is prevented; however, in a *rad9* background, all four mutant strains die rapidly.

Unlike those of *CDC2*, *CDC9*, and *CDC17*, the function of *CDC13* is not yet understood. The sequence, reported here, for the *CDC13* gene does not suggest a biochemical function. The DNA lesions left in *cdc13* mutants display two properties in a *rad9* background that distinguish them from the lesions left in *cdc2*, *cdc9*, and *cdc17* mutants. First, the maximum temperature at which cells containing the *cdc13-1* allele can grow (the maximum permissive temperature) is 28°C in a *rad9* background and 25°C in *RAD9*. This implies that the *RAD9* check-

point is particularly sensitive to the kind of damage that results when Cdc13p fails to function, since it arrests the cell cycle at a sublethal level of damage. *cdc17 rad9* and *cdc9 rad9* double mutants have lower maximum permissive temperatures than *cdc* single mutants (66). Second, *cdc13 rad9* mutant cells shifted from the permissive temperature to the restrictive temperature continue to divide at the same rate as *CDC13 rad9* cells for about three generations even though division is accompanied by rapid loss of viability, with each cell producing a microcolony of about 20 dead cells at the restrictive temperature. *cdc17 rad9* and *cdc9 rad9* double mutants divide once after being shifted to an elevated temperature and arrest in the next cell cycle (66). The lesions remaining after a failure of *cdc2*, *cdc17*, or *cdc9* function must either be immediately lethal or be recognized by another checkpoint system that does not depend on the *RAD9* gene. The damage left by failure of Cdc13p function, on the other hand, must not trigger a second checkpoint function and is therefore specific for the *RAD9* checkpoint system. Therefore, identification of the lesion remaining after a *cdc13* defect may identify the specific signal for the *RAD9* pathway. The fact that *cdc13 rad9* cells eventually arrest at the restrictive temperature is probably due to the loss of essential genes since, as we report in this paper, *cdc13 rad9* cells experience DNA loss at high temperature.

The DNA lesions remaining in a *cdc13* mutant differ from those left by *cdc2*, *cdc9*, and *cdc17* in a third characteristic. DNA damage introduced randomly into the genome spontaneously or by X-irradiation induces mitotic recombination uniformly along the chromosome (14, 39). Lesions remaining in *cdc2* and *cdc9* mutants also induce mitotic recombination uniformly. Cdc17p deficiency induces recombination about twice as frequently in the half of the chromosome distal from the centromere. In contrast, Cdc13p deficiency induces recombination exclusively at the ends of chromosomes. This result suggests that Cdc13p may function uniquely at telomeres.

Telomeres define a special domain of the chromosome with unique properties. Yeast telomeres consist of many repeats of simple G₁₋₃T::C₁₋₃A repeats. As in other organisms, yeast telomeres are arranged so that the GT repeats are on the 5'-to-3' DNA strand oriented from centromere to telomere (54). The telomeric sequences serve to distinguish natural chromosome ends from ends produced by chromosome break-

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TABLE 1. Yeast strains used in this study

Yeast strain		Genotype	Source
Diploid	Haploid		
9056 through 9062		<i>MATa cdc13</i> or <i>cdc13</i> <i>MATα cdc13 CDC13</i> <i>can1 ura3</i> + YIp5 B610H-4.4R:: <i>CAN1</i> <i>can1 ura3 hom3</i>	This study
	7859-10-2a	<i>MATa CDC13 RAD9 leu2 trp1 ura3</i>	This laboratory
	7859-7-1a	<i>MATa CDC13 rad9::LEU2 leu2 trp1 ura3</i>	This laboratory
	7845-7-4a	<i>MATa cdc13-1 RAD9 LEU2 trp1 ura3</i>	This laboratory
	7845-7-1a	<i>MATa cdc13-1 rad9::LEU2 leu2? trp1</i> <i>ura3 can1 cyh2 ura1</i>	This laboratory
	4078-13-1b	<i>MATα cdc13-1 his7 trp1 ura3</i>	This study
	4078-13-1a	<i>MATa cdc13-1 his7 leu2 ura3</i>	This study
	5559-3-3b	<i>MATα ste7-2 ade2 trp1</i>	This laboratory
1340-5a		<i>MATa HO thr4</i> <i>MATα HO thr4</i>	Yeast Stock Center
9069	4078-13-1a	As listed above for 4078-13-1a	This study
	6727-3-1b	<i>MATα his3 ura3 leu2</i>	This study
9086	9077-1-1b	<i>MATα his3 leu2 trp1</i>	This study
	9077-1-2a	<i>MATa his3 leu2 ura3</i>	This study
9087	9077-1-3b	<i>MATα his3 leu2 rad9::HIS3</i>	This study
	9077-1-3a	<i>MATa his3 leu2 rad9::HIS3</i>	This study
9086D13		<i>MATa CDC13 leu2 RAD9 his3</i> <i>MATα cdc13::LEU2 leu2 RAD9 his3</i>	This study
9087D13		<i>MATa CDC13 leu2 rad9::HIS3 his3</i> <i>MATα cdc13::LEU2 leu2 rad9::HIS3 his3</i>	This study
9127	4078 13-3a	<i>MATa cdc13-1 his7 leu2 ura3 can1</i>	This laboratory
	4053-3-4b	<i>MATα CDC13 his7 trp1 ura3</i>	This laboratory
9029	9123-17-3a	<i>MATa cdc13-1 rad9::TRP1 leu2 trp1 ura3</i>	This study
	9123-2-3b	<i>MATα CDC13 rad9::TRP1 leu2 ura3</i>	This study

age. Telomeres, unlike broken chromosomes, do not undergo fusion (reviewed in reference 2), and normal chromosome ends are not subject to degradation, in contrast to ends lacking telomeres (52).

Telomeres form the substrate for novel mechanisms of DNA replication which may require functions unnecessary for replication of other chromosomal regions. Because DNA replication in the 3'-to-5' direction requires short RNA primers, removal of the terminal RNA primer would be expected to result in continuous shortening of the chromosome. Many organisms contain a specialized terminal transferase, telomerase, which extends the GT-rich strand (5' to 3') (56). *S. cerevisiae* has recently been shown to contain telomerase activity (15, 42) and a gene for telomerase RNA (57). Telomerase activity may account for the transient appearance of the short stretch of single-stranded GT-rich DNA at telomeric ends that has been observed during S phase (67). Gene conversion between the telomeric repeats of different chromosomes may also play a role in maintaining chromosome length (48).

Telomeres also affect the expression and replication of adjacent DNA. Normally active genes inserted near telomeres become transcriptionally silenced (22). In addition, telomeres affect the timing of nearby DNA replication origins (6, 20). *ARSS01*, an origin of replication located 25 kb from the right end of chromosome V, fires late in S phase in its normal location but early in S phase on a circular plasmid. On a small linear plasmid, where it is again near a telomere, it fires late.

Moreover, insertion of the early-acting origin *ARS1* near a telomere on chromosome V converts it to a late-acting origin.

In this paper, we examine the response of telomere-associated DNA to a deficiency of Cdc13p function. We made use of the fact that *cdc13 rad9* cells divide several times at elevated temperature to examine the DNA that is synthesized under restrictive conditions. The results suggest roles for Cdc13p in telomere-associated DNA replication or stability and for single-stranded DNA in activating the *RAD9* checkpoint. We also report the cloning, sequencing, and mapping of *CDC13*.

MATERIALS AND METHODS

Yeast and bacterial strains and plasmids. Congenic strains of *S. cerevisiae* were constructed for use in this study by backcrossing genes from other strain backgrounds 10 times with the A364A strain background. Deletion mutations were introduced into the A364A background by integration of DNA that had been modified in vitro. The *cdc* mutations were originally isolated in A364A. The original isolates of the *cdc* mutants were backcrossed at least five times with strains congenic to A364A to remove any cryptic mutations that might have been introduced by the mutagenesis. Plasmid isolation was carried out in *Escherichia coli* RR1 [*leuB gal lac xyl ara pro supE44* ($r^- m^-$); from B. Hall], *E. coli* JF1754 and TB1 [*araD (lac-proAB) rspL f80 lacZΔM15* ($r_K^+ m_K^+$); from T. Baldwin] were also used. The yeast strains used in this study are listed in Table 1.

Standard yeast and bacterial genetic methods were used (37, 55). The plasmids used in this study are listed in Table 2.

Cloning. *CDC13* was cloned from a library of yeast genomic DNA in YE24 (12) by complementation of the temperature-sensitive allele *cdc13-1*. Plasmids from eight independent transformants were examined by restriction mapping. They showed five different restriction patterns but with several fragments in

TABLE 2. Plasmids used in this study

Plasmid	Insert	Source or reference
YIp5	Yeast integrating vector	4
YE24	Yeast multicopy vector	4
YE13	<i>LEU2</i>	8
pCAN1-2-1	<i>CAN1</i>	27
B610H-4.4R	Chromosome V fragment E ^a in YIp5	17
BG151	<i>CAN1</i> in B610H-4.4R	This study
pBG132	Chromosome V fragment A ^a in YIp5	This study
pBG149	Chromosome V fragment B ^a in YIp5	This study
pBG136	Chromosome V fragment C ^a in YIp5	This study
pBG145	Chromosome V fragment D ^a in YIp5	This study
YRp131B	6.6-kb Y' subtelomeric repeat	14
pGT23	<i>ARS2</i>	29
pVZ1	Sequencing vector	S. Henikoff
pBG120	<i>LEU2-ARS2</i> in pVZ1	This study
pBG121	<i>CDC13</i> in pBG120, orientation 1	This study
pBG122	<i>CDC13</i> in pBG120, orientation 2	This study
pBG152	<i>cdc13::LEU2</i>	This study
pHR85-31	Chromosome V fragment flanked by phage T3 and T7 RNA promoters	D. E. Gottschling
pRI316	<i>Cen UR43</i> vector	F. Heffron
pBG168	<i>CDC13</i> on a 4.5-kb <i>ApaI</i> fragment in pRS316	This study

^a See Fig. 1.

common. We concluded that they represented overlapping fragments of the same region of the genome. Subsequent work was carried out with the smallest plasmid, designated YE24-*CDC13*-161-4. YE24-*CDC13*-161-4 was integrated into the genome of the [cir⁰] yeast strain 4009-2-1b by continuous culture under selection for uracil prototrophy. Integrants appear on medium lacking uracil as faster-growing papillations on the lawn of plasmid-containing cells. A clone in which uracil prototrophy had become stable was crossed to a *cdc13-1* mutant. The genetic linkage of the integrated *URA3* gene of the vector to the *CDC13* locus was determined by sporulation and microdissection. We found no nonparental ditypes or tetratypes among 17 tetrads in which ts:TS and *ura3::URA3* assorted 2:2, indicating that the site of integration of the plasmid was tightly linked to the *CDC13* locus.

To confirm that the gene we cloned was indeed *cdc13* and not a high-copy-number suppressor, we ligated a 4.5-kb *ApaI* fragment containing the complementing activity into pRS316, a centromere-containing plasmid, to create pBG168. In single copy, the cloned gene was still able to complement the temperature sensitivity of a *cdc13-1* mutant. We also transformed pRS316 and pBG168 into the *cdc13::LEU2/CDC13 RAD9/RAD9* and *rad9/rad9* diploids described below. After sporulation and dissection, the diploids transformed with the vector alone showed 2:2 segregation of viability and no *Leu*⁺ spores were recovered. The diploids carrying vector plus insert gave tetrads with three and four viable spores and *Leu*⁺ spores were recovered (data not shown), demonstrating that the cloned gene can complement the deletion in single copy.

Construction of the vector used in sequencing. pGT23 was cut with *XhoI* to release a 0.6-kb fragment carrying *ARS2*. The fragment was ligated into YE13 that had been partially cut with *SalI*. Transformants were screened for a *BglII* restriction pattern consistent with ligation of the fragment at the *SalI* site adjacent to *LEU2*. The plasmid was digested with *BglII* to liberate a 3-kb fragment carrying both *LEU2* and *ARS2*. This fragment was end filled and cut with *HpaI* to create a 2.8-kb blunt-ended molecule. The sequencing vector pVZ1 was partially cut with *SspI* and ligated with the *LEU2-ARS2* fragment. One of the potential sites of integration disrupted the phage origin of pVZ1. The resulting plasmids were screened by restriction mapping for one in which the phage origin was intact. This was designated pBG120.

Sequencing. A 4.5-kb *ApaI* fragment from YE24-*CDC13*-161-4 was ligated into the *ApaI* polylinker site of pBG120 in both orientations to create pBG121 and pBG122. Sets of nested deletions were created from pBG121 and pBG122 by limited exonuclease III degradation (27). To localize the gene within the insert, we transformed the deletion plasmids into the yeast strain 4078-13-3a and tested their ability to complement the *cdc13* defect. *Leu*⁺ transformants were streaked for single colonies at 23 and 36°C, and the colony size was compared to that produced by cells transformed with the undeleted plasmids pBG121 and pBG122.

DNA sequencing was done by the dideoxy method (53). Polymerization was carried out with the modified T7 DNA polymerase Sequenase (61) according to procedures supplied by U.S. Biochemical Corp. The reactions were analyzed on 6% acrylamide gels in a buffer gradient of 0.5 to 2.5× TBE (31).

Construction of a *CDC13* deletion. pBG121 was cut with *NruI* and *SauI* to

excise a fragment from -107 to +2414 of the open reading frame. *LEU2* was derived from YE13 on an *EcoRV-HpaI* fragment. The *LEU2*-containing fragment was isolated from an agarose gel along with several other fragments too close in size to be resolved from it. This mixture of fragments was ligated into *NruI-SauI*-cut pBG121 and transformed into *E. coli* RR1, a strain carrying the *leuB* mutation which is complemented by *LEU2*. Plasmids from *LEU*⁺ bacterial transformants were screened by restriction mapping. The *ApaI* fragment from a plasmid with the correct restriction pattern was ligated into pVZ1 and confirmed to carry *LEU2* by complementation of *leuB*. The pVZ1 construct was designated pBG152. The *ApaI* fragment carrying *cdc13::LEU2* was isolated from pBG152 and transformed into the diploid yeast strain 9039 (*MATa/MATa cdc13-1/CDC13 leu2/leu2*) (35). Both TS⁺ and ts⁻ *LEU*⁺ transformants were obtained. To test the effect of *rad9* on the phenotype of a *cdc13* deletion, the fragment containing *cdc13::LEU2* was transplanted into 9086 (*CDC13/CDC13 RAD9/RAD9*) and 9087 (*CDC13/CDC13 rad9::HIS3/rad9::HIS3*). Integration of the fragment at the *CDC13* locus was confirmed by Southern blot analysis (44).

Monitoring the induction of chromosome loss and mitotic recombination. (i) Strain construction. A 4.4-kb *EcoRI* fragment from the telomere-proximal region of the right arm of chromosome V (38) was ligated into YIp5 to create B610H-4.4R. *CAN1* on a 4-kb *XhoI* fragment (9) was ligated into the *XhoI* site of B610H-4.4R to create pBG151. pBG151 was singly cut with *KpnI* and inserted into chromosome V to create a duplication of the chromosomal sequences. YIp5 can be excised from the chromosome by an intrachromosomal recombination between the repeated chromosomal sequences. Excision can occur in such a way as to leave the sequence that was originally in the plasmid and remove the chromosomal sequence. By plating on 5'-fluoroorotic acid, we selected clones which had undergone excision of YIp5 (3). Many of these "pop-outs" were found to be canavanine sensitive, indicating that the region containing the *CAN1* insert had been retained. The structure was confirmed by genomic Southern analysis. The genotype of this strain, I-pBG151, was *MATa cdc13-1 his7 leu2 ura3-52 can1 B610H-4.4R::CAN1*. I-pBG151 was crossed to a series of strains in which YIp5 had been inserted at known locations on chromosome V. These diploids were sporulated and dissected to produce strains with *CAN1* and YIp5 on the same chromosomal arm. Diploids were then constructed with the following genotype:

$$\frac{a \text{ cdc13}}{\alpha \text{ cdc13}} \left(\begin{array}{c} \text{CDC} \\ \text{or} \\ \text{CDC} \end{array} \right) \frac{can1 \text{ ura3 } hom3 \text{ YIp5 B610H-4.4R::CAN1}}{can1 \text{ ura3} +}$$

(ii) Measuring induction of chromosome loss and recombination. Duplicate or triplicate cultures were used in each experiment. Cultures were grown to mid-log phase at 23°C in defined medium lacking uracil to select for maintenance of the YIp5 insertion. Cell density was adjusted to 5 × 10⁶ cells per ml, and uracil was added to 60 µg/ml. The cultures were divided into two aliquots. One was incubated at the permissive temperature for 7 h, and the other was incubated at 36°C for 3.5 h and then at the permissive temperature for 3.5 h to allow time for phenotypic expression. Both cultures were then plated on synthetic medium with 60 µg of uracil per ml and with or without 60 µg of canavanine per ml. The frequency of canavanine resistance increased 15- to 40-fold in the portion of the culture incubated at the restrictive temperature compared to that in the aliquot which remained at the permissive temperature.

Drug-resistant colonies were replicated in medium lacking uracil or methionine to determine which recessive markers had become homozygous in each clone. The frequency of drug resistance for the whole culture was multiplied by the percentage of events occurring in a given genetic interval to calculate the number of events occurring in that interval. The number of events occurring in that interval in the unshocked culture was subtracted from the number of events in the same interval in the shocked culture to give the number of induced events in that interval.

DNA isolation and Southern blots. Yeast DNA used for two-dimensional agarose gels and slot blots was isolated from isolated nuclei and subjected to Hoechst/CsCl gradients (34). DNA for other Southern blots was isolated by vortexing cells with glass beads in the presence of phenol-chloroform (29). Two-dimensional gels were run as described previously (7, 8). Gels were blotted to Hybond-N (Amersham) for Southern hybridizations.

Hybridizations with DNA probes were performed in 0.5 M sodium phosphate (pH 7.2)-1 mM EDTA-7% sodium dodecyl sulfate (SDS)-100 µg of tRNA per ml (Sigma) at 65°C. The same hybridization conditions were used for DNA that had been transferred to Hybond-N from one- or two-dimensional gels and for slot blots of native and denatured DNAs. Southern hybridizations with RNA probes were carried out in 6× SSC (pH 7) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-2× Denhardt's reagent-0.1% SDS-100 µg of tRNA per ml at 68°C (51). Filters were prehybridized with carrier for 1 to 2 h before the probe was added. Hybridizations were carried out for 16 to 20 h.

DNA probes were made by random-primed synthesis by using [α-³²P]dATP (3,000 Ci/mmol; New England Nuclear) (18). RNA probes were transcribed from pHR85-31 that had been cut with *SalI* (T7 RNA polymerase) or *NotI* (T3 RNA polymerase). They were labeled with [α-³²P]ATP (3,000 Ci/mmol; New England Nuclear) (51).

Determination of the amount of single-stranded DNA in native DNA by using slot blots. For each slot, 1 µg of native DNA was adjusted to 165 µl in distilled water. Then, 15 µl was removed, made 0.2 M in NaOH, and incubated at 65°C for 30 min to denature the DNA. Native samples and denatured aliquots were

TABLE 3. Genetic mapping of *CDC13*: summary of two crosses^a

Cross	Asci				Distance (centimorgans)
	Total	Parental	Nonparental	Tetratypes	
<i>cdc13/ste7</i>	67	27	1	39	33
<i>cdc13/HO</i>	36	31	0	5	7
<i>ste7/HO</i>	36	15	2	19	44

^a *CDC13* was mapped in a two-point cross relative to *STE7* and in a three-point cross relative to *STE7* and *HO*. Cells carrying the *ste7-2* mutation are able to mate at 23°C but are sterile at 34°C (21). The *HO* gene causes cells to switch mating type with high efficiency (reviewed in reference 20). We found that it was possible to score *STE7* and *HO* in the same cross because *HO ste7-2* spores switch mating type but self-mate with low efficiency at 23°C so that an *HO ste7* spore gives rise to a colony that contains both *MATa* and *MATα* cells, while *HO STE7* colonies are nonmating and *ho ste7* cells mate monosexually. *ho STE7* and *ho ste7* spores can be distinguished because the latter are sterile at 34°C.

brought to a 300-μl final volume with 10× SCP (1 M NaCl, 0.3 M Na₂HPO₄, 0.02 M EDTA, adjusted to pH 6.8 with HCl). The native DNA and denatured aliquot were drawn through a Hybond-N membrane with a Schleicher and Schuell Minifold II Slot Blotter. Each slot was washed three times with 300 μl of 10× SCP. The DNA was fixed to the membrane by exposure to 1.2 mJ of UV light. The filters were kept moist to minimize denaturation of any bound native DNA. Filters were probed only once. Hybridization conditions are described above. Hybridization was quantified with a Molecular Dynamics PhosphorImager. The percentage of single-stranded DNA in the native sample was calculated as hybridization to the native sample compared to the hybridization to the denatured aliquot.

Quantitative Southern blots. DNA was denatured and filtered through a Hybond-N membrane as described above. Quadruplicate filters were prepared. The filters were challenged with a series of DNA probes. Filters were stripped for rehybridizations by incubation in 200 ml of 0.4 M NaOH at 50°C for 30 min, followed by 2× 200 ml of 0.1× SSC–0.1% SDS–0.2 M Tris-HCl (pH 7.5) for 15 min. Hybridization conditions are described above. Hybridization was quantified with a Molecular Dynamics PhosphorImager. To compare the amount of binding for different DNA samples, we calculated the ratio of binding for the probe to that of the centromere-proximal probe *URA3*. To normalize the results, the ratio for each sample was divided by the average value of the quadruplicate samples of *CDC13 RAD9* DNA.

Nucleotide sequence accession number. The *CDC13* sequence has been registered with GenBank (accession number M76550).

RESULTS

Cloning, sequencing, and mapping of *CDC13*. The *CDC13* gene was localized to a 4.5-kb genomic fragment by its ability to complement the temperature-sensitive phenotype of *cdc13-1*. The predicted protein contains 924 amino acids and has a molecular mass of 104,903 kDa. It has no significant homology to other reported protein sequences and no recognized structural motifs. *CDC13* was mapped to the left arm of chromosome IV, between *STE7* and *HO* (Table 3).

CDC13 was shown to be a unique gene by genomic hybridization (data not shown). However, Northern (RNA) blots of poly(A) RNA from logarithmically growing wild-type *S. cerevisiae* yielded two transcripts of 3.1 and 4.6 kb (data not shown). We found no indication of cell cycle control at the transcriptional level since both transcripts were present in RNA isolated from a variety of *cdc* mutants arrested at the G₁, S, or G₂ stage of the cell cycle (data not shown).

The deletion phenotype. To determine whether the temperature-sensitive phenotype represented the null phenotype of the *CDC13* gene, a 2.7-kb fragment (–107 to +2414) from the cloned gene was excised and replaced with *LEU2*. The *cdc13::LEU2* construct was transformed into congenic *RAD9/RAD9* and *rad9::HIS3/rad9::HIS3* diploids (35). The Leu⁺ diploids were sporulated and dissected. Viability segregated 2:0 in both strain backgrounds, and all viable spores were Leu[–]. We infer that *CDC13* is an essential gene and that the lethality of the

TABLE 4. Number of buds formed by *cdc13* spores after germination^a

Strain	Genotype	No. of spores	Avg no. of buds
9027	<i>cdc13-1/CDC13 RAD9/RAD9</i>	16	2.4 ± 0.6
9086D13	<i>cdc13::LEU2/CDC13 RAD9/RAD9</i>	13	2.9 ± 1.0
9129	<i>cdc13-1/CDC13 rad9/rad9</i>	10	17 ± 9
9087D13	<i>cdc13::LEU2/CDC13 rad9/rad9</i>	11	29 ± 11

^a Diploids heterozygous for the temperature-sensitive *cdc13-1* mutation and homozygous for *RAD9* or *rad9::HIS3* were sporulated at 36°C. Diploids heterozygous for the deletion were sporulated at 23°C. After 24 h, the arrested spores were micromanipulated apart and counted. Each cell body was counted as a bud, so that an unbudded cell was counted as one bud and a large-budded cell was counted as two buds. Buds of *CDC13* spores were too numerous to count at that time.

deletion does not result from irreversible arrest at the *RAD9* checkpoint.

For comparison, diploids heterozygous for the *cdc13-1* allele were sporulated and dissected and the spores were placed at 36°C. The inviable spores were examined microscopically. In the *RAD9* background, both *cdc13::LEU2* and *cdc13-1* gave rise to one or two enlarged large-budded cells. This result indicates that very little Cdc13p from the *CDC13* parent is being inherited by the *cdc13* spore. In the *rad9* background, cells arrested with unbudded, small-budded, and large-budded morphology. Microcolony size from 10 *cdc13-1 rad9* spores ranged from 8 to 35 cells and from 11 *cdc13::LEU2 rad9* spores from 8 to 44 cells (Table 4). Since the *cdc13-1* temperature-sensitive allele behaves essentially the same as the *cdc13* deletion allele in either the *RAD9* or *rad9* background, we conclude that the *cdc13-1* temperature-sensitive allele displays a null phenotype.

Epistasis of *cdc13* and *cdc17*. First cycle arrest of both *cdc13* and *cdc17* temperature-sensitive mutants depends on *RAD9*. *cdc13 rad9* strains continue to divide for several generations, while most *cdc17 rad9* cells divide once after being shifted to the restrictive temperature. This may indicate that division without completion of the Cdc17p-dependent step results in damage that triggers a second checkpoint or that arrests the cell cycle directly, while failure to complete the Cdc13p-dependent step gives rise to damage that is specific for the *RAD9* checkpoint. In this case, we would expect *cdc17*-induced damage to be epistatic to *cdc13*-induced damage. To determine whether the *cdc13* or *cdc17* phenotype was epistatic, we examined single and double *cdc* mutants in a *rad9* background. Cells growing at permissive temperature were plated on solid me-

TABLE 5. No. of buds in microcolonies of *cdc13-1* and *cdc17-1 rad9* mutants plated at the restrictive temperature^a

Genotype and time	% of microcolonies with indicated no. of buds						
	1	2	3	4	5–9	10–14	≥15
<i>cdc13 rad9</i> (0 h)	13.5	77.5	5.0	3.0	1.0	0.0	0.0
<i>cdc13 rad9</i> (8 h)	0.5	4.5	0.5	0.5	0.5	5.5	88.5
<i>cdc17 rad9</i> (0 h)	10.0	77.5	5.5	4.5	2.5	0.0	0.0
<i>cdc17 rad9</i> (8 h)	0.0	1.5	0.5	6.0	53.5	26.5	12.0
<i>cdc13</i> (0 h)	8.5	68.0	7.0	13.5	3.0	0.0	0.0
<i>cdc17 rad9</i> (8 h)	1.5	3.0	1.0	11.5	61.5	14.5	7.0

^a Logarithmically growing cultures were sonicated and plated on prewarmed YEPD plates. The number of buds per microcolony was scored immediately or after 8 h of incubation at 36°C. Each cell body was counted as a bud, so that an unbudded cell was one bud and a large budded cell was two buds, etc. Two hundred microcolonies were scored.

FIG. 1. Induced mitotic recombination on chromosome V-R in *cdc13-1/cdc13-1* diploids. *CAN1* was integrated into the right arm of chromosome V, 3 kb proximal to the X and Y' subtelomeric repeats. Cloned sequences, indicated by boxes A to D, were used to target the insertion of YIp5 at various distances to the *CAN1* gene. A set of diploids (9056 through 9062) was constructed, homozygous or heterozygous for *cdc13* and having the following configuration of chromosome V: *can1/can1 ura3/ura3 +/hom3 YIp5(URA3) CAN1*. Logarithmically growing cultures were incubated at 36°C for 3.5 h and then grown to stationary phase (three to four generations) at 23°C before plating on medium containing canavanine. The y axis represents the percentage of total recombinational events that occur centromere proximal to the site of YIp5 insertion in a particular strain, i.e., the portion of canavanine-resistant colonies that are also uracil prototrophs. This percentage was determined by replica plating the canavanine-resistant colonies to medium lacking uracil. *hom3* was scored by replica plating to medium lacking methionine. The values obtained for unshocked cultures were subtracted from the shocked cultures. In *cdc13/CDC13* heterozygotes, there was no significant increase in the frequency of canavanine resistance (data not shown).

dium at restrictive temperature. After 8 h at restrictive temperature, almost 90% of *cdc13 rad9* cells formed microcolonies of greater than 15 cells, while only 12% of the *cdc17 rad9* cells formed microcolonies of that size. The distribution of microcolony sizes in the *cdc13 cdc17 rad9* triple mutant was very similar to that observed for the *cdc17 rad9* double mutant (Table 5), indicating that *cdc17* is epistatic to *cdc13*.

Cdc13p deficiency induces recombination exclusively near the ends of chromosomes. When cells carrying the temperature-sensitive allele *cdc13-1* were incubated at a semipermissive temperature, mitotic recombination was induced in telomere-proximal regions of chromosome VII (14) and chromosome II (40). In order to examine the distribution of *cdc13*-induced events more precisely in relation to the physical map and also to extend our observations to another chromosome, we integrated the *CAN1* gene 3 kb from the X subtelomeric element of chromosome V-R. YIp5, an integrating plasmid carrying the *URA3* marker, was then inserted at various distances centromere proximal to *CAN1* to create a set of strains in which YIp5 insertions marked the terminal 60 kb of the 480-kb arm (19). The strains were also heterozygous for *hom3*, a centromere-proximal marker that was used to monitor recombination events and chromosome loss in regions distant from the telomere. Over 80% of the recombination events induced by heat shock of the *cdc13/cdc13* diploid strain occurred within the region covered by the YIp5 insertions (Fig. 1). The frequency of recombination events was approximately inversely related to

distance from the telomere within this region. Less than 0.5% of the induced *Can^r* clones were also *hom3*, indicating that few recombination events were induced near the centromere and that little or no chromosome loss occurred.

Cdc13p is not required for the activity of telomere-proximal origins of replication. Several lines of evidence imply that telomeres have long-distance effects on chromosome structure in *S. cerevisiae*. Genes inserted near telomeres may be transcriptionally silenced (22) and less available to enzymatic modification (21). Telomere-associated origins are activated late in S phase but may become activated early when transferred to a plasmid (19, 45). Special factors may be necessary for activation of origins of replication that lie within the sphere of influence of telomeres. If Cdc13p played such a role, it might explain the limitation of *cdc13*-induced recombination to telomere-associated regions of the chromosome. In order to test this possibility, we examined DNA from *CDC13* and *cdc13* cells at the restrictive temperature for *cdc13* by two-dimensional electrophoresis. This technique separates DNA replication intermediates from the bulk of DNA and distinguishes regions that contain an origin of replication from those which are replicated by forks originating elsewhere (7, 8). *ARS501* is ideally placed for such an experiment. Lying 30 kb from the telomere of chromosome V-R, it is within the region where recombination is induced in *cdc13-1* homozygous diploids (Fig. 1). Moreover, it fires late in S phase, and its timing is controlled by its proximity to the telomere (20). Log-phase cultures of *CDC13 RAD9*, *CDC13 rad9*, *cdc13 rad9*, and *cdc13 RAD9* were shifted from 23 to 36°C for 4 h. *CDC13 RAD9*, *CDC13 rad9*, and *cdc13 rad9* cells continued to divide logarithmically during this time; *cdc13 RAD9* cells arrested as large-budded cells. After 4 h, DNA was isolated from all four cultures, digested with *Xba*I, and displayed on a two-dimensional agarose gel. The DNA was blotted and hybridized to a DNA probe corresponding to region D (Fig. 1) which labels the *Xba*I

FIG. 2. Late origins continue to function at the restrictive temperature in *cdc13 rad9* cells. Logarithmically growing cultures were shifted from 23 to 36°C for 4 h. All cultures continued to divide exponentially except *cdc13 RAD9*, which arrested in *G*₂. DNA from each culture was digested with *Xba*I, subjected to two-dimensional electrophoresis, and blotted. The blots were hybridized with *ARS501*. (A) *CDC13 RAD9*; (B) *CDC13 rad9*; (C) *cdc13 rad9*; (D) *cdc13 RAD9*. The diagram indicates the identities of the different DNA structures as determined by Brewer and Fangman (7, 8) and this paper. In this gel, we see some trailing of signal in the first dimension from the main spot of hybridization in all four samples. We believe this to be an artifact since it occurs in some but not all gels. We have not indicated it in the diagram identifying the DNA species. The scales indicate the position of molecular weight markers (MW; size given in kilobases) in the first dimension.

FIG. 3. DNA from *cdc13 rad9* cells dividing at 36°C was digested with *Xba*I. It was then incubated with no enzyme (A), DNase-free RNase (B), or mung bean nuclease (C) prior to being subjected to two-dimensional electrophoresis and blotted. The blot was hybridized with probe D (Fig. 1), which labels *ARS501*.

fragment containing *ARS501* (Fig. 2). DNA from the dividing cells, i.e., *CDC13 RAD9*, *CDC13 rad9* and *cdc13 rad9*, contained replication bubbles, indicating that *ARS501* is functioning as an origin of replication in all these cells. Since *ARS501* is active in *cdc13 rad9* cells at the restrictive temperature, we conclude that *cdc13* is not required to initiate DNA synthesis at this telomere-proximal origin. DNA from the arrested *cdc13 RAD9* cells shows neither replication origins nor replication forks. This argues that the *RAD9* checkpoint does not arrest cells in late S stage by stopping the progression of replication complexes but probably arrests in G₂ after completion of DNA synthesis.

***cdc13* mutants accumulate single-stranded DNA at chromosome ends.** The two-dimensional gel of DNA from *cdc13 rad9* cells grown at 36°C and probed with *ARS501* revealed a heterodisperse species of hybridizing material that was not present in DNA isolated from the *CDC13* strains examined (Fig. 2C). Two lines of evidence indicated that this species was predominantly single-stranded DNA: its nuclease sensitivity and its electrophoretic mobility. To examine the nuclease sensitivity of the unusual species, we treated the DNA with mung bean nuclease, which degrades single-stranded polynucleotides (41), or with DNase-free RNase A prior to running it on a two-dimensional gel and blotting. The gel was blotted and hybridized to probe D as before (Fig. 3). While the bulk of the hybridizing material was resistant to digestion by these enzymes, the unusual species was destroyed by mung bean nuclease. It was resistant to RNase A, consistent with its identification as primarily single-stranded DNA.

To examine the electrophoretic pattern of a heterodisperse band of single-stranded DNA, we digested yeast DNA with *Xba*I to create a spectrum of fragment sizes. A portion of the digest was heat denatured to generate single-stranded DNA. The native and denatured samples were run on two-dimensional gels, either separately or combined. Denatured DNA runs differently from native DNA, and its electrophoretic properties resemble those of the species revealed by hybridization to DNA from *cdc13 rad9* cells (Fig. 4).

By blotting and hybridization, we found that a 3.5-kb fragment had an apparent size of 2.5 kb when denatured (Fig. 4C). Assuming single-stranded molecules run at about 70% of their length when compared to double-stranded molecular weight standards, we estimate that the single-stranded DNA from *cdc13 rad9* cells might be as large as 17 kb. However, it is not clear this material is entirely single stranded. Digestion by restriction enzymes of DNA with a single-stranded gap or tail might release a molecule with double-stranded ends but with enough single-stranded character to affect its electrophoretic properties. However, since unrestricted DNA from *cdc13 rad9*

cells reveals a heterodisperse band of size and intensity similar to those present after restriction digestion, much, if not all, of the single-stranded DNA must exist as a free species, not covalently bound to large regions of double-stranded DNA (data not shown).

Formation of single-stranded DNA in *cdc13* cells is limited to telomere-proximal regions. To determine whether the formation of single-stranded DNA in *cdc13 rad9* cells was limited to telomere-proximal regions and whether it formed only at the restrictive temperature for *cdc13*, we divided a culture of *cdc13 rad9* cells growing logarithmically at 23°C and shifted part of it to 36°C. After 4 h, DNA was isolated from both 23 and 36°C portions. DNA was isolated, digested with the appropriate restriction enzyme, and displayed on two-dimensional gels. The blots were hybridized to probes containing either the telomere-proximal origin *ARS501* or centromere-proximal origin of replication *ARS1* (Fig. 5). The *ARS501* probe revealed the single-stranded DNA from the portion of the culture incubated at 36°C (Fig. 5D), but not from the portion that remained at 23°C (Fig. 5C). The *ARS1* probe did not hybridize to single-stranded DNA from either temperature (Fig. 5A and B). We conclude that the formation of single-stranded DNA is limited to telomere-proximal regions and that its appearance is caused by failure of Cdc13p to act.

DNA from *cdc13 RAD9* did not exhibit single-stranded DNA when probed with *ARS501* (Fig. 2D), but both *cdc13*

FIG. 4. The electrophoretic patterns of native and denatured DNAs. DNA from a *CDC13 RAD9* strain was digested with *Xba*I. Part of the digest was heat denatured. It was subjected to two-dimensional electrophoresis and photographed. (A) Native DNA only. (B) Native plus denatured DNA. (C) Gel B was blotted and hybridized to probe D (Fig. 1). The dark arrow indicates the region of hybridization to native DNA. The white arrow indicates the region of hybridization to the denatured DNA. The scale indicates the position of molecular weight markers (MW; size given in kilobases) in the first dimension.

FIG. 5. *cdc13 rad9* mutants do not accumulate single-stranded DNA corresponding to the centromere-proximal origin *ARS1* at 36°C or to telomere-proximal *ARS501* at 23°C. A logarithmic culture of *cdc13 rad9* cells growing at 23°C was divided, and half was shifted to 36°C. After 4 h, DNA was isolated, digested with *NheI* (A and B) or *XbaI* (C and D), subjected to two-dimensional electrophoresis, and blotted. A fragment containing *ARS1* was hybridized to DNA from cultures at 23°C (A) or 36°C (B). Probe D (Fig. 1), which labels the restriction fragment containing *ARS501*, was hybridized to DNA from cultures at 23°C (C) or 36°C (D). The diagram in Fig. 2 identifies the different DNA components of the blot. In this gel, we see some trailing of signal in the second dimension from the main spot of hybridization in all four samples. We believe this to be an artifact since it occurs in some but not all gels. We have not indicated it in the diagram identifying the DNA species. The scale at the top indicates the molecular size in kilobases in the first dimension.

RAD9 and *cdc13 rad9* cells at 36°C showed a band of single-stranded DNA when probed with a fragment from the Y' subtelomeric repeat (data not shown). We conclude that single-stranded DNA is present in *cdc13 RAD9* cells and may be limited to regions very close to the telomere. This interpretation is consistent with the quantitation of the amount of single-stranded DNA in native DNA from *cdc13 RAD9* and *cdc13 rad9* cells by hybridization of radioactive probes described below.

Quantitation of the amount of single-stranded DNA in *cdc13* cells. The amount of single-stranded DNA was quantitated by Southern blotting (17, 59) (Fig. 6). The chromosome V sequences used for making the hybridization probes in these experiments were the same as those used to target the insertions marking chromosome V for the recombination study described in Fig. 1. In addition, *URA3* was used as a centromere-proximal probe, and a fragment from the Y' subtelomeric repeat (16) was used as a probe for regions very close to the telomere. ³²P-dATP-labeled synthetic poly(GT-AC) was used to detect telomeric sequences. Because only the AC-rich strand of the probe was labeled, only the GT-rich telomeric strand was monitored in these experiments. The *CDC13 RAD9* and *CDC13 rad9* strains showed less than 1% of hybridization to any of the probes tested (Fig. 6). For the centromere-proximal sequence *URA3*, the *cdc13* strains showed the same low levels of hybridization as the *CDC13* strains. For the telomere-proximal sequences, however, the *cdc13* strains show an elevated level of hybridization. *cdc13 RAD9* cells contained elevated levels of filter-binding material corresponding to the most telomere-proximal regions but not from the *ARS501* region. This result is consistent with our observations on the two-dimensional gels, where DNA from *cdc13 RAD9* cells lacked single-stranded DNA when probed with *ARS501* but did show it when probed with the Y' subtelomeric repeat.

To confirm that the material binding to the filter was indeed

single-stranded DNA, we subjected the samples to nuclease digestion before carrying out the filter-binding protocol (Table 6). DNA from *cdc13 rad9*, *cdc13 RAD9*, and *CDC13 RAD9* cells that had been shifted to 36°C prior to harvest was mixed with the appropriate reaction buffer. As a control, alkali-denatured DNA was added to one sample of *CDC13 RAD9* DNA as an authentic source of single-stranded DNA. The samples were divided into two aliquots; one was incubated with the single-strand-specific nuclease S1 (62) or with DNase-free RNase A prior to filtering, whereas the other was incubated without enzyme. A portion from each incubation mixture was removed and treated with alkali to monitor the total DNA remaining after enzyme treatment. The filter was hybridized with a fragment from the Y' telomeric element. The filter-binding material in native DNA prepared from all three strains was destroyed by S1 nuclease but not by RNase A (Table 6). This result implies that the material binding to the filter is the same that was observed on the two-dimensional gel. The same enzyme susceptibility was found for the authentic denatured DNA which had been added to the native DNA from *CDC13 RAD9* cells. Most of the DNA was recovered after enzyme digestion of the native samples, showing that the double-stranded DNA was not susceptible to digestion.

FIG. 6. *cdc13* cells contain elevated levels of single-stranded DNA in telomere-proximal regions. Logarithmically growing cultures of *CDC13 RAD9*, *CDC13 rad9*, *cdc13 RAD9*, and *cdc13 rad9* cells were shifted to 36°C for 4 h. DNA was isolated and filtered through a nylon membrane as described in Materials and Methods. The graph shows the amount of binding to the native DNA samples, expressed as a percentage of the binding observed when the DNA was denatured prior to filtration. Below the x axis are the positions on chromosome V of the DNA probes used in this experiment (compare with Fig. 1). Probe Y' is a fragment of the Y' subtelomeric repeat containing the Y' *ARS* (16). The telomeric probe was made from poly(GT-AC). It should be noted that probes *URA3* and A to E are unique sequences and label chromosome V specifically, but probe Y' hybridizes to repeated sequences found at the ends of most chromosome arms and poly(GT-AC) hybridizes to telomeres but also to linkers between the subtelomeric repeats and to GT-AC repeats located internally in chromosomes. Therefore, the amount of single-stranded DNA detected by the Y' and GT-AC probes represent an average for many chromosome ends and may not reflect the amount of telomeric single-strandedness of chromosome V-R. For probes A, C, D, E, and Y', percentages from three independent DNA isolations are averaged. For *URA3* and GT-AC only two independent determinations were made. For *cdc13 RAD9*, only one determination was made with *URA3*.

TABLE 6. Nuclease sensitivity of the filter-binding material in native DNA^a

Strain and growth conditions (h, °C)	Enzyme	Binding native/denatured (%) ^b	Total DNA recovered (%) ^c
<i>cdc13 rad9</i> (2, 36)	No S1 nuclease	6.7	100
	+ S1 nuclease	0.0	91
	No RNase A	5.1	100
	+ RNase A	5.1	98
<i>cdc13 RAD9</i> (2, 36)	No enzyme	2.2	100
	+ S1 nuclease	0.0	83
	No RNase A	1.8	100
	+ RNase A	1.7	92
<i>CDC13 RAD9</i> (4, 36)	No enzyme	0.2	100
	+ S1 nuclease	0.0	96
	No RNase A	0.2	100
	+ RNase A	0.2	94
<i>CDC13 RAD9</i> + 10% denatured DNA	No enzyme	9.7	100
	+ S1 nuclease	0.1	98
	No RNase A	8.4	100
	+ RNase A	6.5	124

^a DNA was mixed with the appropriate reaction buffer and divided into two aliquots. Enzyme was added to one aliquot, and the same volume of water was added to the other. After incubation for 30 min at 37°C, one-tenth of each sample was removed and denatured. Native and denatured samples were brought to 300 µl of 10× SCP and filtered through a nylon membrane. Data are the averages of triplicates.

^b Binding of native DNA, expressed as a percentage of binding for each sample when the DNA had been denatured prior to filtration. The probe used was Y', a 2.5-kb fragment containing the *ARS* of the Y' subtelomeric repeat. Compare with Fig. 1.

^c The average of the three samples not treated with enzyme was set to 100%. Recovery of total DNA in the enzyme-treated samples was normalized to that value.

When a culture of logarithmically growing *cdc13 rad9* cells is shifted to the restrictive temperature, exponential growth continues for at least 6 h, although loss of viability begins within 2 h. To see how the amount of single-stranded DNA in *cdc13 rad9* cells changed as a function of time, DNA was isolated and Southern blots were performed on native DNA with a series of probes. Hybridization to telomere-proximal probes was maximal at 2 h after the shift and decreased thereafter (Table 7). The *ARS501* region 30 kb from the end of chromosome V showed a smaller increase in single-strandedness than regions closer to the telomere and was maximal at 4 to 6 h after shift-up. Regions 60 and 45 kb from the end of chromosome V did not increase in single-strandedness over the course of the experiment. By 8 h, replication had stopped and the amount of single-strandedness for all probes decreased almost to control

TABLE 7. Appearance of single-stranded DNA in *cdc13 rad9* cells as a function of time after shift to 36°C for a series of chromosomal regions

Hours at 36°C	% Single-strandedness ^a (± %) for region:					
	<i>URA3</i>	A	D	E	Y' telomeric repeat	GT-AC
0	0.8 ± 1.1	0.8 ± 0.3	0.6 ± 0.1	0.2 ± 0.1	0.8 ± 0.7	0.8 ± 0.4
2	0.3 ± 0.2	1.3 ± 1.2	1.9 ± 0.1	6.2 ± 0.8	6.5 ± 4.0	6.4 ± 1.9
4 ^b	0.4 ± 0.0	1.3 ± 0.7	4.3 ± 0.4	5.1 ± 0.9	4.9 ± 1.6	5.0 ± 2.2
6	0.5 ± 0.0	0.8 ± 0.5	4.6 ± 0.2	2.5 ± 1.0	2.6 ± 0.4	2.6 ± 0.8
8	0.1	1.0 ± 0.1	1.9 ± 0.2	2.0 ± 1.4	1.8 ± 1.0	1.6 ± 0.1

^a Calculated as the amount of filter-binding material in native DNA compared to the amount when the DNA is denatured prior to filtration. DNA regions A, D, and E are the same as in Fig. 1.

^b Data for the four-hour time point appeared in Figure 6 for *cdc13 rad9* cells.

TABLE 8. Hybridization^a of strand-specific probes^b to native DNA isolated from cells which had been incubated at 36°C for 4 h

Genotype	% Hybridization	
	3'-to-5' strand (centromere to telomere)	5'-to-3' strand (centromere to telomere)
<i>CDC13 RAD9</i>	0.3	0.3
<i>CDC13 rad9</i>	0.4	0.3
<i>cdc13 RAD9</i>	0.9	2.9
<i>cdc13 rad9</i>	0.5	8.9

^a Hybridization is expressed as binding to native DNA as a percentage of the binding to denatured DNA.

^b RNA probes were transcribed from the plasmid pHR85-31, which contains a 3-kb fragment of DNA flanked by promoters for T3 and T7 RNA polymerases. The insert is from a region 13 kb from the end of chromosome V-R, just proximal to region E of Fig. 1. The transcript from the T3 promoter complements the 3'-to-5' strand, centromere to telomere. The transcript from the T7 promoter complements the 5'-to-3' strand.

levels. These results indicate that the telomere-proximal regions are in a dynamic state with the appearance of single-stranded DNA occurring transiently.

Single-stranded DNA corresponds to the GT-rich telomere strand. To determine whether the single-stranded DNA observed in *cdc13* mutants was strand specific, we performed Southern blots of native DNA with strand-specific RNA probes (51). Only the probe complementary to the 5'-to-3' strand, centromere to telomere, hybridized to native DNA from *cdc13* strains grown at the elevated temperature at levels higher than *CDC13* strains (Table 8). Both transcripts hybridized when the DNA was denatured before being drawn through the nylon membrane. Little hybridization with either transcript to native DNA from *cdc13* strains grown at the permissive temperature was observed (data not shown). We conclude that the single-stranded component of native DNA in *cdc13* strains is primarily from the 5'-to-3' strand in centromere to telomere direction, which terminates in G₁₋₃T repeats.

Chromosome ends are lost during proliferation of *cdc13 rad9* mutant cells. In order to determine whether chromosome ends are stable in *cdc13 rad9* cells during proliferation at 36°C, we examined total genomic DNA by quantitative Southern analysis (17). DNA from *CDC13 RAD9* cells served as a control (Table 9). DNA was denatured before being bound to a nylon membrane. It was then hybridized to a series of DNA probes. The ratio of hybridization to telomere-proximal sequences to the centromere-proximal sequence *URA3* was normalized to the value of that ratio for *CDC13 RAD9* cells. There was no loss of sequence A, located 60 kb from the telomere, from *cdc13 rad9* cells. Telomere-proximal sequence E and the Y' subtelomeric repeat were reduced by 40%. Sequence D, 25 kb proximal to the telomere, was reduced by 20%. The loss of DNA correlated well with the regions that appeared as single-stranded DNA.

DISCUSSION

The *CDC13* gene product is needed for telomere integrity. Deletion of the *CDC13* gene results in cell death, indicating that Cdc13p is involved in an essential function. Two observations suggest that Cdc13p performs a role in DNA replication or maintenance. First, mitotic recombination is induced by temperature stress in *cdc13* temperature-sensitive cells. Mitotic recombination is a repair process induced by the presence of damaged DNA and is frequently enhanced in cells with mutations in DNA replication (25). Second, the arrest in G₂ is dependent on the *RAD9* checkpoint (66). Rad9p is a compo-

TABLE 9. Ratio of telomere-proximal sequences to a centromere-proximal sequence decreases in DNA from cells dividing at the restrictive temperature^a

Genotype and hours at 36°C	Ratio in region:			
	A ^b	D ^b	E ^b	Y' telomeric repeat
<i>CDC13 rad9</i> , 4	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
<i>cdc13 rad9</i>				
0	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1
2	1.1 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
4	1.1 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
6	1.1 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
8	1.1 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.6 ± 0.1

^a Genomic DNA was denatured prior to binding to the nylon membrane. Therefore, these numbers represent the total amount of material corresponding to the probes. The ratio of the indicated sequence to the centromere-proximal sequence containing *URA3* is normalized to the average value of that ratio for *CDC13 RAD9*.

^b Same as in Fig. 1.

ment of a signal transduction pathway that detects damage or incomplete replication and inhibits mitosis until repair or replication is completed (64). It seems likely, therefore, that failure to complete the *CDC13* function results in damaged or incompletely replicated DNA.

Damage-induced DNA recombination occurs near the site of damage (46, 58). Temperature-induced recombination in *cdc13-1* cells had been reported to occur preferentially in telomere-proximal regions of the chromosome (14, 40). On chromosome V-R, where we created a series of genetic markers at known positions on the physical map, recombination was induced almost exclusively in the 60 kb closest to the telomere and the frequency increased with proximity to the telomere.

These results suggest that Cdc13p might play a role in replication or maintenance of sequences at or near the telomere. In order to examine DNA synthesized in *CDC13* and *cdc13-1* at the permissive and restrictive temperatures, we took advantage of the fact that *cdc13 rad9* cells continue to replicate DNA for several generations after being shifted to the restrictive temperature. Replication intermediates were normal in *cdc13 rad9* cells from *ARS501*, an origin located near the telomere on chromosome V-R. However, a new species of DNA appeared that was neither present at the permissive temperature for *cdc13-1* nor at the elevated temperature for *CDC13* cells. The electrophoretic mobility and nuclease susceptibility of this unusual species were consistent with single-stranded DNA. The presence of single-stranded DNA was more pronounced at the telomere and decreased with distance from the telomere, in general agreement with the profile of induced recombination. Quantitation of the amount of total DNA revealed a loss of telomere-proximal sequences relative to internal DNA sequences, but the amount lost was far less than that expected if telomere replication was entirely arrested at the restrictive temperature. This result is consistent with our observation that *ARS501* continues to function. These results suggest that telomere replication is occurring but that the product is incomplete or unstable. Other genes necessary for telomere maintenance are *EST1* (43), which encodes a subunit of the telomerase (42), and *TLC1*, which encodes the telomerase RNA (57). However, *est1* and *tlc1* mutants continue to grow normally for at least 30 generations (42, 43). This implies that division in the absence of *EST1* and *TLC1* does not provoke the *RAD9* checkpoint and that the loss of telomeres in *cdc13* mutants is not due to the failure of telomerase.

Another gene known to affect telomere length is *CDC17*. This gene codes for an essential DNA polymerase (14). Incubation of cells carrying the temperature-sensitive allele *cdc17-1* at the highest temperature at which they are able to sustain growth results in progressive elongation of their telomeres (14). The phenotype of *cdc17-1* resembles that of *cdc13-1* in several ways. Brief incubation of *cdc17-1* cells at the restrictive temperature induces mitotic recombination preferentially near the telomere, although induced recombination is not limited to telomere-proximal regions as in *cdc13-1* (13, 40). The G₂ arrest phenotype of *cdc17-1* is dependent on *RAD9* at 34°C, although at 38°C *cdc17-1* cells arrest randomly in the cell cycle in a *RAD9*-independent manner (13, 66). Incubation of *cdc13-1* cells at their maximum permissive temperature causes a small increase in average telomere length though not as large as in *cdc17-1* cells at their maximum permissive temperature (13). Although *cdc13* and *cdc17* display these similarities, there are also significant differences. Mitotic recombination is induced in *cdc17* cells over a larger area of the chromosome, as mentioned above, and *cdc17-1 rad9* cells undergo a single division after being shifted to the restrictive temperature while *cdc13-1 rad9* cells continue to divide for several generations. In *cdc13 cdc17 rad9* triple mutants, the *cdc17* phenotype is epistatic to that of *cdc13*. This result may indicate that passage of cells through mitosis without having completed the *CDC17* function results in damage that provokes another checkpoint or arrests the cell cycle directly. The damage left in *cdc17* cells at high temperature might be different from that in *cdc13* cells or wider in distribution along the chromosome. This putative checkpoint might explain the *RAD9*-independent arrest of *cdc17-1* cells at 38°C. The damage in *cdc13* cells or its limitation to regions near telomeres may be a specific signal to the *RAD9* checkpoint.

Possible roles for Cdc13p. The most striking consequence of Cdc13p deficiency is the appearance of single-stranded DNA uniquely at telomeric regions corresponding to the GT-rich strand. Any model for Cdc13p function must account for this fact, and we present several possibilities. In the first scenario, Cdc13p functions in telomere replication. In *S. cerevisiae*, at least 30 base pairs of GT telomeric sequence become single-stranded for a short time at the end of S phase (67). It has been suggested that this is a normal intermediate of telomere replication. It is not known whether this GT extension represents elongation of the 5'-to-3' (centromere to telomere) strand or degradation of the 3'-to-5' (centromere to telomere) strand. The normal role of Cdc13p might be to limit elongation or prevent degradation. In either case, an abnormal telomere resulting from lack of Cdc13p might be a substrate for a 5'-to-3' exonuclease.

In a second model, Cdc13p is needed uniquely or is more critical in telomere-associated regions for lagging strand DNA replication. Telomere-proximal DNA replication occurs late in S phase (6, 19) because of its proximity to the telomere (20), and it may be that special proteins are needed to replicate this portion of the genome.

Finally, Cdc13p may function to mask telomeres from the DNA repair system. The telomeres of linear plasmids have been found to associate briefly at the completion of DNA replication (26, 67). It has been suggested that this association is necessary to mask the telomeric ends from the *RAD9* checkpoint control (67). Cdc13p might be necessary for this association, or in more general terms, Cdc13p might be necessary to distinguish telomeric ends from ends created by DNA double-strand breaks. DNA double-strand breaks are susceptible to attack by 5'-to-3' exonucleases during recombination and repair (59, 60).

The signal for the *RAD9* checkpoint. The *RAD9* checkpoint serves to arrest the cell cycle in G_2 when DNA damage is present or when DNA replication is incomplete (1, 64, 66). What aspect of damaged or incompletely replicated DNA is recognized as the signal to promote cell cycle arrest? The fact that *cdc13* mutant cells accumulate single-stranded DNA at the restrictive temperature suggests that single-stranded DNA might be the signal. Single-stranded DNA is an intermediate in many repair pathways, including double-strand break repair (59), excision repair (28), and recombinational repair (50), and would be present at the fork of an incomplete replicon. Since single-stranded DNA is an intermediate in many repair processes, one detection system would then be able to respond to many kinds of damaging agents. The SOS system of *E. coli* arrests cell division in response to the presence of single-stranded DNA (reviewed in reference 68), and single-stranded DNA arrests mitosis in *Xenopus* extracts (39).

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