Stn1, a new *Saccharomyces cerevisiae* protein, is implicated in telomere size regulation in association with Cdc13

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We have isolated *STN1*, an essential *Saccharomyces cerevisiae* gene, as a suppressor of the *cdc13-1* mutation. A synthetic lethal interaction between a temperature-sensitive mutant allele of *STN1*, *stn1-13*, and *cdc13-1* was observed. Stn1 and Cdc13 proteins displayed a physical interaction by two-hybrid analysis. As shown previously for *cdc13-1*, *stn1-13* cells at the restrictive temperature accumulate single-stranded DNA in subtelomeric regions of the chromosomes, but to a lesser extent than *cdc13-1* cells. In addition, both Cdc13 and Stn1 were found to be involved in the regulation of telomere length, mutations in *STN1* or *CDC13* conferring an increase in telomere size. Loss of Stn1 function activated the *RAD9* and *MEC3* G₂/M checkpoints, therefore confirming that DNA damage is generated. We propose that Stn1 functions in telomere metabolism during late S phase in cooperation with Cdc13.

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Telomeres are specialized structures at the ends of eukaryotic chromosomes that are necessary for maintaining chromosomal integrity (for review, see Gilson et al. 1993; Greider 1995; Zakian 1995a,b). One of the essential functions of telomeres is to protect chromosome ends from end-to-end fusions and degradation (McClintock 1941; Sandell and Zakian 1993). Another essential function of telomeres is to provide a mechanism for the replication of chromosome ends, a mechanism that requires a specialized enzyme, telomerase, therefore preventing a progressive shortening and degradation of the chromosomes (for review, see Greider 1995; Zakian 1995b). Telomeres also have nonessential functions, such as to repress the transcription of adjacent genes and to affect the timing of nearby DNA replication origins (Gottschling et al. 1990; Ferguson and Fangman 1992; Brewer et al. 1993; for review, see Sandell and Zakian 1992). Telomeres associate with one another, assembling unusual chromatin structures, called telosomes, which probably have a role in regulation of transcription in these regions (for review, see Gilson et al. 1993; Gilson and Gasser 1995).

The telomeric DNA of almost all eukaryotic cells is composed of short repeat sequences, which in *Saccharo-*

myces cerevisiae are heterogeneous C₁₋₃A/TG₁₋₃ repeats ~300 bp long. Telomere length is thought to be the result of a complex balance between processes that lead to their degradation and processes that lengthen them (for review, see Zakian 1995b). The 300 bp-long C₁₋₃A/TG₁₋₃ telomeric sequences of *S. cerevisiae* are thought to be necessary and sufficient for all essential functions of telomeres. TG₁₋₃ tails are overhangs of the telomeric G-rich strand that represent natural intermediates during telomere replication forming at the end of S phase (Wellinger et al. 1992). In addition to serving as a substrate for telomere repeat addition by telomerase and therefore G-rich strand elongation, TG₁₋₃ tails are thought to serve as a substrate for putative telomere-binding proteins (McEachern and Blackburn 1995; for review, see Zakian 1995b). However, TG₁₋₃ tails were found to be present on telomeres in strains lacking telomerase. Moreover, in vivo-generated DNA molecules were found to have TG₁₋₃ tails at both ends, a situation incompatible with the existence of a sole telomerase-dependent mechanism of formation of TG₁₋₃ tails (Wellinger et al. 1996). Conventional replication mechanisms and telomerase-mediated strand elongation ultimately yield molecules with the leading strand of synthesis having a blunt end and the other end having a short 3' overhang. This observation, together with the demonstration that *cdc13* mutant cells display an increased amount of single-stranded DNA in telomeric and subtelomeric regions of their chromosomes, has led to

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the proposition that a strand-specific exonuclease might generate TG₁₋₃ tails at each telomere (Wellinger et al. 1996) and that cell cycle-controlled degradation of the C₁₋₃A strand might be regulated by Cdc13 (Garvik et al. 1995). Therefore, Cdc13 might limit the extent of exonuclease digestion of the TG₁₋₃ tails or limit their elongation. In agreement with this model, it was shown that mutation of *RAD24* in a *cdc13* mutant background was sufficient to suppress the generation of extra single-stranded DNA seen in *cdc13* single mutants (Lydall and Weinert 1995). Interestingly, Rad24 appears to control Rad17, a putative exonuclease (Lydall and Weinert 1995). In *S. cerevisiae*, *RAD24* and *RAD17* are part of a complex system of checkpoint control that ensures that cells do not enter mitosis in the presence of damaged or unreplicated DNA by blocking, by an unknown mechanism, cell cycle progression (Weinert and Hartwell 1988, 1993; Weinert et al. 1994). DNA damage generated by mutations in *CDC13* or other S phase genes (such as *CDC2*, *CDC8*, *CDC9*, or *CDC17*, for instance) is detected readily by the checkpoint system which, in addition to *RAD17* and *RAD24*, also comprises *RAD9*, *MEC1*, *RAD53* (*MEC2*), and *MEC3* (Weinert and Hartwell 1988, 1993; Weinert et al. 1994).

In studies on telomeres, the role of Cdc13 seems to be of particular importance because it is a good candidate not only for controlling the elongation of TG₁₋₃ tails and subsequently telomere maintenance but also for association between telomeres and protection against the *RAD9*-dependent DNA repair system (Garvik et al. 1995). The *CDC13* gene has been cloned, but its sequence has not suggested a biochemical function (Garvik et al. 1995). Recently, Cdc13 was shown to bind to several single-strand G-rich yeast telomeric oligomers (Nugent et al. 1996). Together with the observations of telomere shortening in *cdc13-2^{est}* mutant cells and of a genetic interaction between *CDC13* and *EST1*, a regulatory component of yeast telomerase, this has led to the proposition that Cdc13 might mediate telomerase loading at the chromosome end (Nugent et al. 1996). Moreover, the absence of an enhanced phenotype in a strain bearing a disruption of *TLC1*, the telomerase RNA gene, and the *cdc13-2^{est}* mutation, and, in contrast, the observation of an enhancement of the senescence phenotype associated with telomere shortening in the *cdc13-1 Δtlc1* double mutant suggested a dual role for Cdc13 in telomere maintenance (Nugent et al. 1996). One function of Cdc13, lost in the *cdc13-2^{est}* mutant, would be to positively regulate telomerase activity by mediating its access to the telomeric G-rich extension. A second function of Cdc13, lost in the *cdc13-1* mutant, would be to protect the chromosome end from degradation (Garvik et al. 1995; Nugent et al. 1996).

In this context, studies on Cdc13-dependent pathways are fundamental for understanding telomere metabolism and its relationship to the cell cycle. We report here the isolation of a novel, essential, gene of *S. cerevisiae*, *STN1*, as a suppressor of the *cdc13-1* mutation. We show that both *cdc13-1* and *stn1-13* loss-of-function mutants exhibit a defect in telomere size regulation that leads to

a dramatic elongation of telomeres. These mutants were also found to accumulate increased amounts of single-stranded DNA in subtelomeric regions of their chromosomes. Finally, Cdc13 and Stn1 scored positive for interaction by two-hybrid analysis, suggesting that they form a complex in vivo. Based on these findings, we propose a model in which Cdc13 is a dual regulator (alternatively, a positive and a negative regulator) of telomerase activity. We further propose that Stn1 acts as an inhibitor of telomerase loading through its interaction with Cdc13. In our model, Cdc13 mediates access of the telomerase complex, Tlc1/Est1, to the chromosome end but also mediates access of Stn1 (or activation of its inhibitory activity) to this complex, resulting in turning off of telomerase activity.

Results

Isolation of STN1

At the restrictive temperature of 37°C, *cdc13-1* mutants arrest in G₂. At the semi-permissive temperature of 30°C, *cdc13-1* mutants grow very slowly, reflecting partial activation of the *RAD9*-dependent checkpoint. This checkpoint is thought to allow repair of *cdc13*-induced chromosomal damage before progression through mitosis (Weinert and Hartwell 1993; Garvik et al. 1995). Under such conditions, genomic DNA fragments from a low-copy plasmid bank capable of suppressing the *cdc13-1* mutation could potentially identify proteins interacting specifically with Cdc13.

Strain *cdc13-1* was transformed with a *S. cerevisiae* genomic DNA library constructed in YCp50 (Rose et al. 1987) and ~60,000 transformants were screened for their ability to complement the slow growth phenotype of *cdc13-1* cells at 30°C and inability to restore complete growth at 37°C (to avoid re-isolation of *CDC13*). Two colonies were isolated and their plasmids recovered. Partial restriction mapping demonstrated that both were identical, bearing the same 7-kb insert. Subcloning revealed that a 3.5-kb DNA fragment containing a single 1.7-kb-long open reading frame (ORF) with no apparent intronic sequences was capable of suppressing the *cdc13-1* mutation (data not shown). This gene was named *STN1*, for suppressor of *cdc* thirteen. A search of the compiled data bases using the BLAST program at the National Center for Biotechnology Information (NCBI), National Institutes of Health (NIH), revealed that the sequenced ORF was a novel gene exhibiting no homology with any known protein or gene and no consensus sequences with previously identified motifs. After the *STN1* gene was sequenced, a DNA sequence identical to *STN1* appeared in the data banks as a result of the automated sequencing program of chromosome IV, which corresponded to ORF YDR082w, gene D4456.

To confirm the structure of the *STN1* gene, its cDNA was isolated following transformation with a *S. cerevisiae* cDNA library (see Materials and Methods) of a strain in which the *STN1* gene had been disrupted and that was kept alive by the presence of an *STN1*-URA3

plasmid that was subsequently shuffled out on 5-FOA medium (see below; see also Materials and Methods). Sequencing of this cDNA confirmed the primary structure of the *STN1* coding sequence, in particular the identity of the initiating ATG and terminating stop codons and confirmed the absence of introns in the genomic DNA (data not shown).

STN1 is an essential gene

A diploid yeast strain was transformed with a DNA fragment containing the *STN1* ORF interrupted by the *TRP1* gene from the ATG to the *EcoRI* site at position 842, leaving 642 nucleotides downstream, as described in Materials and Methods. Sporulation and tetrad analysis of this heterozygous [*STN1/stn1::TRP1*] diploid strain showed that only two of the four spores per tetrad were viable at 30°C on YEPD (in all 52 tetrads dissected). All viable spores were *Trp*[−], indicating that the inviable spores contained the *stn1::TRP1* replacement. Microscopic examination indicated that mutant spores, in which the *STN1* gene had been disrupted, germinated, and completed one to three rounds of division before arresting with a nonuniform phenotype (data not shown). Actual disruption of *STN1* was verified by PCR and Southern analysis. Re-introduction of *STN1* on a centromeric plasmid restored viability to the *stn1* disrupted strain (data not shown). Another *STN1* disruption was constructed by replacing nucleotides 1–559 with the *URA3* locus (see Materials and Methods). Sporulation of the *stn1::URA3/STN1* diploid gave a 2::2 viability, all viable spores being *Ura*[−]. Therefore, wild-type *S. cerevisiae* cells are incapable of growth in the absence of the *STN1* gene and arrest following spore germination with a nonhomogeneous morphology.

Suppression by overexpression of STN1 is specific for the cdc13 mutation

Suppression of *cdc13-1* by YCp50–*STN1* did not occur at 32°C or higher temperatures (data not shown). However, when *STN1* was maintained in the cell carried on a YEp (high copy) plasmid, rescue of *cdc13-1* was observed at 32°C, but not higher (data not shown). Finally, *STN1* expressed from a YCp–*GAL* plasmid (a centromeric plasmid in which the *STN1* gene was cloned behind the strong inducible *GAL1* promoter) could rescue the *cdc13* mutation at 34°C, but not higher (Fig. 1). Therefore, overexpression of *STN1* cannot totally bypass the *CDC13* function, suggesting that *Stn1* is not simply a downstream element in the *Cdc13* pathway.

To assess the specificity of *cdc13* suppression, strains bearing mutations in *CDC2* (DNA polymerase III; Boulet et al. 1989), *CDC9* (DNA ligase; Johnston and Nasmyth 1978), or *CDC17* (a structural subunit of DNA polymerase α ; Carson and Hartwell 1985) were transformed with YEp–*GAL*–*STN1*. None of the defects conferred by these mutations was rescued by overexpressed *STN1* (data not shown).

Figure 1. Suppression of the temperature-sensitive growth phenotype of the *cdc13-1* mutation by overexpression of *STN1*. YNG32 [*cdc13-1*] was transformed with YCp111–*GAL* (left) or YCp111–*GAL*–*STN1* (right) and 10-fold serial dilutions (from left to right in each row) of a representative transformant grown for 2 days at the indicated temperatures on galactose-based medium are shown.

Analysis of Stn1 depletion and of the stn1-13 mutation

Initially, before a temperature-sensitive mutant of *STN1* was available, we used the *stn1::TRP1* disruptant that was kept alive owing to a YCp–*GAL*–*STN1* (*URA3*) plasmid. Shifting such cells from galactose to glucose medium resulted in progressive exhaustion of *Stn1*. Under these conditions, it was not until after 1 to 2 days, a delay presumably necessary for total depletion of intracellular stores of previously accumulated *Stn1* protein, that a defect in cell growth was observed. Cells became very large and appeared to arrest with a large bud (Fig. 2A). However, some cells continued to grow very slowly (Fig. 2A). Because the *GAL1* promoter is tightly repressed by glucose, we interpreted this result as indicating that some endogenous promoter-like sequences in the coding region of the *STN1* gene were driving its expression independently of the *GAL1* promoter. To verify this hypothesis, the *stn1::TRP1* YCp–*GAL*–*STN1*–*URA3* strain was transformed with a YCp–*LEU2* (promoterless) plasmid carrying *STN1* ORF without any endogenous promoter sequences, and the transformed cells forced to lose the YCp–*GAL*–*STN1*–*URA3* plasmid on 5-FOA medium. The appearance of *Ura*[−] colonies indicated that expression of the *STN1* gene could be driven by putative promoter-like sequences within the coding region of the gene or cryptic promoter-like sequences in the vector. Under such conditions, however, expression of *STN1* was at a lower level than when driven by the real, physiological promoter, as growth was impaired seriously (data not shown). In the absence of the YCp–*STN1*–*LEU2* plasmid, the *stn1::TRP1* YCp–*GAL*–*STN1*–*URA3* strain was incapable of forming colonies on 5-FOA glucose-based medium, which confirmed that in the previous experiment the *STN1* ORF alone without its promoter was responsible for the growth of the *STN1* disruptant strain (data not shown).

A temperature-sensitive mutation of *STN1* was isolated using PCR under mutagenic conditions. The mutagenized PCR products were then transformed into a *STN1* disruptant strain containing a YCp–*GAL*–*STN1*–

Figure 2. (A) Arrest phenotype of *Stn1*-depleted cells. Strain YNG346 bearing a disruption of *STN1* and kept alive owing to the presence of a plasmid containing *STN1* under the control of the *GAL1* promoter was grown on a galactose plate. Cells were then streaked onto a glucose plate. After 48 hr on glucose, most cells have become arrested with large buds. Other cells continue to grow very slowly on glucose because of the presence of promoter-like sequences in the *STN1* ORF (see text). (B) Strain YNG429, disrupted for *STN1*, and containing a temperature-sensitive allele of *STN1*, *stn1-13*, displays a severe growth defect, visible here after 6 hr at 37°C on a plate, with large-budded cells accumulating. The appearance of slow-growing colonies most probably corresponds to leakiness of the mutation. Bar, 10 μ m. (C) The three rows represent 10-fold serial dilutions of cells from strain YNG429 which were grown overnight at the indicated

temperatures. (D) *stn1-13* and *cdc13-1* mutations are synthetically lethal. Spores bearing both the *cdc13-1* and *stn1-13* mutations (see text for explanations) grew only very slowly at 25°C, and stopped dividing completely at 30°C, whereas singly mutant cells were capable of proliferation at 30°C. The two patches of cells in each row represent 10-fold serial dilutions of the same culture.

URA3 plasmid, together with a linear YCp-LEU2 plasmid bearing *STN1* flanking regions at its extremities, a process known as the gap repair method (see Materials and Methods). After loss of the *STN1*-URA3 plasmid on 5-FOA, one temperature-sensitive allele of *STN1*, *stn1-13*, was selected. We do not know yet whether the *stn1-13* phenotype corresponds to one or several mutations. Although *stn1-13* cells could grow well at temperatures between 25°C and 32°C, they grew slowly at 34°C and displayed a severe growth defect at 37°C (Fig. 2B,C). The appearance of colonies after several days at 37°C indicated that the *stn1-13* mutation was conferring a severe growth defect rather than a total growth arrest (Fig. 2B). We noted that all cells were growing very slowly at 37°C, although to varying degrees, and that, clearly, formation of colonies was not attributable to a reversion of the mutation in some cells. It is therefore probable that the *stn1-13* mutation is somewhat leaky.

To more precisely determine the arrest point of the *stn1-13* mutant, cultures were first synchronized with the antimicrotubule agent, nocodazole, and then released at the restrictive temperature; cell cycle progression was monitored at subsequent intervals. In both controls and *stn1-13* mutant cells at 30°C as well as at 37°C, release from the nocodazole block was followed within 30–45 min by a rapid drop in the percentage of cells with large buds that indicated exit from M phase accompanied by separation of mother and daughter cells (Fig. 3A). This demonstrated that the *STN1* execution point is located before the nocodazole block point which, in *S. cerevisiae*, is in late G₂ or at the G₂/M transition (Jacobs et al. 1988). To establish whether this arrest occurred before or after the completion of S phase, cells were blocked in S phase with hydroxyurea. Both wild-type cells at either

temperature and *stn1-13* at their permissive temperature of 30°C exited mitosis synchronously starting at ~85 min after release from the S-phase block (Fig. 3B). On the other hand, at the restrictive temperature of 37°C, *stn1-13* cells remained in S or G₂ and did not enter into a new cell cycle.

We conclude from both the nocodazole and hydroxyurea experiments that the *stn1-13* mutation arrests cells during late S phase or during G₂ before the nocodazole-sensitive step. Immunofluorescence of microtubules demonstrated that both *stn1-13* and *cdc13-1* mutants displayed an arrest phenotype characterized by the presence of a mitotic spindle of medium size, typical of G₂ arrest (Fig. 3C).

Synthetic lethal interaction between cdc13-1 and stn1-13

Given that *STN1* had been isolated as a suppressor of the *cdc13-1* mutation, we looked for additional genetic interactions between *STN1* and *CDC13*. A strain containing the *cdc13-1* mutation was crossed with the strain containing both a disruption of *STN1* (*TRP1* marker) and the ts *stn1* mutation on a YCp-LEU2 plasmid. In tetrads in which two of the four spores grew normally at 37°C, we assumed that the wild-type *CDC13* and *STN1* genes had cosegregated. In such tetrads, the two normally growing spores were found to be Trp⁺, and therefore the two spores that could not grow at 37°C were assumed to contain the *cdc13-1* and *stn1-13* mutations. Such spores were found to grow very slowly at 25°C. At 29°C, they could not form colonies, whereas *cdc13-1* and *stn1-13* cells could form colonies at 30°C (Fig. 2D). Therefore,

Figure 3. Arrest caused by the *stn1-13* mutation during the cell cycle. (A) Block and release experiments using nocodazole. Log-phase cultures of wild-type (wt) or *stn1-13* mutant (strain YNG429) were arrested in the presence of 15 μ g/ml of nocodazole [prepared as a stock solution of 10 mg/ml in DMSO] at 30°C for 2 hr and transferred to 30°C (top two panels) or 37°C (bottom two panels) in the presence of the drug for an additional hour. Removal of the drug by centrifugation, while maintaining cultures at 30°C or 37°C, corresponded to time zero [30°C is the optimal temperature of growth for *stn1-13* cells, whereas 37°C is a restrictive temperature for *stn1-13* but not for wild-type cells]. At 15-min intervals, aliquots were fixed with formaldehyde and the percentages of unbudded cells (G_1 , $\blacklozenge$), small and medium buds ($S + G_2 + \text{early M}$, $\blacksquare$) and large buds (mid-M + late M, $\blacktriangle$) were determined microscopically. It should be noted that in *stn1-13* cells percentages of cell cycle arrest by nocodazole (A) or hydroxyurea (B), at 30°C as well as at 37°C, never reached the 85%–90% level attained by wild-type cells at either temperature, but rather stayed around 65%–70%. We believe that, given that the *stn1-13* mutation is deficient in telomere function at all temperatures [see Results corresponding to Fig. 7], it partially activates the DNA damage checkpoint at 30°C (a temperature nevertheless permissive for growth, see Fig. 2) that may delay the accumulation of cells at the drug block. Longer exposures to drugs, capable of increasing the percentage of arrested cells, would have had deleterious effects on cell recovery and were therefore not attempted. (B) Block and release experiments using hydroxyurea. Log-phase cultures of wild-type or *stn1-13* cells were treated with 0.4 M hydroxyurea for 2 hr at 30°C followed by an hour at 30°C (top two panels) or 37°C (bottom two panels) before removal of the drug (time zero). Determination of cell cycle stages at 15-min intervals was as in A. (C) YNG429 (*stn1::TRP1* YCp-*stn1-13*-LEU2) cells (a) were synchronized with 0.4 M hydroxyurea for 3 hr, at 30°C, then released in the absence of the drug at 37°C, fixed 2.5 hr later, and processed for tubulin immunofluorescence labeling and staining of the DNA with DAPI (see Materials and Methods). YNG32 [*cdc13-1*] cells (b), not presynchronized, were incubated at 37°C for 5 hr before being processed as described above.

mutations in the *STN1* and *CDC13* genes display a synthetic lethal interaction.

A mutation in STN1 or depletion of Stn1 activates the RAD9- and MEC3-dependent mitotic checkpoints

It is known from previous work on *cdc13-1* mutants that a failure in *CDC13* function does not lead to immediate arrest in S phase, its presumed execution point, but, instead, activates the *RAD9*-dependent G_2/M checkpoint, and that it is the latter constraint that is responsible for the arrest of *cdc13* cells (Weinert and Hartwell 1993). Consequently, cells with mutations in both *CDC13* and *RAD9* fail to exhibit the pause in G_2 normally imposed by the *RAD9*-dependent checkpoint in the presence of DNA damage, such as that caused by the *cdc13* mutation. Such cells enter into mitosis inappropriately, leading to cell death after an average of three complete cell division cycles (Garvik et al. 1995). Because of the implication of *STN1* in a *CDC13*-related function, we asked whether a lack of *STN1* function in a defective G_2/M checkpoint background would result in a similar mitotic catastrophe phenotype.

When strain NGY359, bearing a disruption of *STN1* (kept alive with YCp-GAL-STN1) and a disruption of *RAD9*, was grown on glucose medium, in order to cause a depletion of Stn1, cells no longer accumulated as large-budded cells as did Stn1-depleted *RAD9*⁺ cells. Rather, they continued to divide and formed microcolonies but rapidly died (data not shown). This phenotype was identical to that observed for *cdc13 rad9* double mutants at 37°C and is indicative of a mitotic catastrophe phenotype (Weinert et al. 1994; Garvik et al. 1995). In the presence of *mec3-2* (N. Grandin and S.I. Reed, unpubl.), a mutation in *MEC3*, another gene involved in mitotic checkpoint control (Weinert et al. 1994), Stn1-depleted cells also continued to divide and formed microcolonies, therefore failing to undergo the arrest observed in *MEC3*⁺ *stn1* cells (Fig. 4). Similarly, a *stn1::TRP1 stn1-13 mec3-2* double mutant placed at 37°C continued to divide actively, forming microcolonies, in contrast to *stn1-13 MEC3*⁺ cells, which at 37°C exhibited a cell cycle delay (Fig. 4). Moreover, when $\Delta rad9 stn1-13$ double mutants were transferred back to 25°C after a 6-hr shift to 37°C, few cells were able to recover, whereas *RAD9*⁺ *stn1-13* and *MEC3*⁺ *stn1-13* cells recovered efficiently (data not shown). A similar loss of viability was observed in strain NGY359 following transfer back to galactose medium after exhaustion of Stn1 by growth on glucose medium.

From these two sets of experiments, we conclude that *stn1-13* cells arrest at the restrictive temperature attributable to the presence of a functional G_2/M checkpoint. Checkpoint-deficient *stn1-13* cells progress through the cell cycle. However, they do so under inappropriate conditions, which apparently leads to cell death by mitotic catastrophe. Because cells with mutations in *RAD9* or *MEC3* that have not incurred DNA damage normally cycle without displaying loss of viability (Weinert and Hartwell 1993; Weinert et al. 1994), we infer that the *stn1-13* mutation or depletion of Stn1 protein causes

Figure 4. Cell cycle arrest attributable to *STN1* loss of function depends on the DNA damage checkpoint. (Top) *stn1* disrupted cells kept alive on galactose medium owing to production of Stn1 protein under *GAL1* promoter control and bearing (*mec3*, right) or not (*MEC3*, left) a mutation in *MEC3*, a DNA damage checkpoint gene, were streaked on glucose medium at 30°C and pictures taken after 3 days (*mec3-2* is not temperature-sensitive). In the presence of the *mec3* mutation (*mec3*, right), cells depleted for Stn1 protein on glucose form numerous microcolonies, a phenotype indicative of a lack of cell cycle arrest by checkpoint control. Conversely, in checkpoint-proficient cells (*MEC3*, left), cells stop progression through the cell cycle. Such cells enlarge because they remain alive and continue to grow while arrested (*MEC3*, left). (Bottom) *stn1*-disrupted cells bearing the temperature-sensitive *stn1-13* mutation and also bearing the wild-type *MEC3* gene (*MEC3*, left) or the *mec3-2* mutant allele (*mec3*, right), were grown at 30°C on plates and transferred to 37°C for 6 hr before pictures were taken. As above, the loss of *STN1* function resulted in the formation of microcolonies in the *stn1-13 mec3-2* double mutant. On the other hand, *MEC3*⁺ cells exhibited a cell cycle delay in G_2 while continuing to grow. Bars, 10 μ m.

DNA damage, which activates the DNA damage G_2/M checkpoint.

The Stn1-13 mutant protein is defective in cdc13-1 rescue even at 30°C

We then asked whether the *stn1-13* mutation specifically affected Cdc13 activity. *cdc13-1* mutant cells were transformed with a multicopy plasmid bearing the *stn1-13* mutant allele or the wild-type *STN1* gene as a positive control, and grown at 25°C before being transferred to various temperatures. Surprisingly, YEp-*stn1-13* was incapable of rescuing the *cdc13-1* mutation at 30°C or 32°C, temperatures fully permissive for the *stn1-13* allele and at which wild-type *STN1* rescues *cdc13-1* on a YEp plasmid (Fig. 5, top panel). However, when the *stn1-13* mutant allele was expressed from a YEp-GAL plasmid, a fairly good rescue of *cdc13-1* was observed at 30°C, a partial rescue was observed at 32°C, whereas no rescue at all was observed at 34°C (Fig. 5, bottom panels). These data demonstrate that overexpressed Stn1-13 mutant protein is clearly defective in its ability to suppress the *cdc13-1* mutation even at a temperature permissive for growth of *stn1-13* mutant cells, but that at extreme levels of expression it can partially rescue the *cdc13-1* mutation. These results suggest that the Stn1-13 protein

Figure 5. Overexpression of the *stn1-13* mutant allele from a YEp plasmid does not rescue *cdc13-1* (top) and from a YEp-GAL plasmid rescues *cdc13-1* partially but less efficiently than overexpression of the *STN1* wild-type allele (bottom). *cdc13-1* YEp vector alone, *cdc13-1* YEp-STN1 and *cdc13-1* YEp-stn1-13 strains were grown at 25°C and then spotted on glucose plates for one day at 30°C. The three patches of cells in each row represent 10-fold serial dilutions of the same culture. *cdc13-1* YEp-GAL vector alone, *cdc13-1* YEp-GAL-STN1 and *cdc13-1* YEp-GAL-stn1-13 strains were grown in glucose medium at 25°C and then spotted on galactose plates for 2 days at the indicated temperatures.

is not fully active, even at temperatures normally permissive for growth, and that cells are sensitized to this defect when they are also defective in Cdc13 function.

CDC13 overexpression does not rescue a STN1 defect

As shown above (Fig. 1), *STN1* overexpression did not rescue the *cdc13-1* mutation at temperatures higher than 34°C. Amplification of *CDC13* using an episomal multicopy plasmid could not rescue the severe growth defect of the *stn1-13* mutation at 37°C (data not shown). It was confirmed that this construct expressed and produced a functional Cdc13 protein, based on its capacity to complement the *cdc13-1* mutation at 37°C (data not shown).

Stn1 and *Cdc13* physically interact in vivo in a two-hybrid system

The fact that *STN1* partially rescued the *cdc13-1* mutation on a low-copy number plasmid, as well as the observation of a synthetic negative interaction between *stn1-13* and *cdc13-1* mutations, suggested that the two proteins Stn1 and Cdc13 form a complex within the cell. In order to examine this possibility, both proteins were expressed simultaneously from plasmids used to detect protein-protein interactions in the yeast two-hybrid system (Fields and Song 1989; Durfee et al. 1993; Harper et al. 1993). Y187 or Y190 *S. cerevisiae* strains were transformed with pAS2-CDC13 or pACT2-STN1 plasmids (see Materials and Methods). Both constructs were functional as confirmed by complementation experiments. The pACT1-STN1 plasmid (pACT1 is identical in structure to pACT2), containing *STN1* cDNA, was isolated by virtue of its capacity to complement the loss-of-function in an *stn1* disruptant strain (see Materials and Methods). Moreover, the presence of pACT2-STN1 in *stn1-13* cells completely suppressed their growth defect at 37°C. Likewise, *cdc13-1* cells transformed with pAS2-CDC13 grew normally at the restrictive temperature of 37°C (data not shown). Neither Cdc13 nor Stn1 chimeras when ex-

pressed alone was found to activate the system (data not shown). On the other hand, when Cdc13 and Stn1 were co-expressed, a strong positive signal was generated, as judged by the rapid accumulation of β -galactosidase activity (intense blue color using X-gal within 1 hr; data not shown). Similarly, diploids formed following conjugation between Y187-pAS2-CDC13 and Y190-pACT2-STN1 rapidly yielded an intense blue color in the X-gal assay, whereas the Y187/Y190 diploids coexpressing *STN1* and *CDC14* (or lamin or p53) displayed a white color (data not shown). The time of appearance of the blue color in the X-Gal assay in cells coexpressing *STN1* and *CDC13* lagged behind that in cells co-expressing *SNF1* and *SNF4*, used as positive standard controls (Fields and Song 1989), only by a few minutes (~40 min vs. ~30 min). This was confirmed by quantitative assays, using ONPG as a substrate, in which β -galactosidase activity was found to be 4.7 and 3.8 nmoles/min per milligram of protein, respectively, in cells co-expressing the *Snf1/Snf4* and the *Stn1/Cdc13* pairs.

stn1-13 mutants, like *cdc13-1* mutants, accumulate single-stranded DNA at telomeres

It was recently shown that *cdc13-1* mutants at restrictive temperature accumulate single-stranded DNA at telomeric and subtelomeric regions of the chromosomes (Garvik et al. 1995; Lydall and Weinert 1995). Similar analyses were conducted on *stn1-13* mutants. DNA was prepared from either *stn1* mutants, *cdc13* mutants, or wild-type cells grown at 25°C and shifted at 37°C for 5 hr. DNA was prepared in such a manner that minimum of DNA breakage was generated (see Materials and Methods). Native DNA or denatured DNA was transferred and membranes incubated with a Y' 32 P-labeled subtelomeric probe. The amount of hybridization to native DNA versus denatured DNA was measured. At 37°C, *stn1-13* mutant cells accumulated single-stranded DNA in subtelomeric regions, although to a lesser extent than *cdc13-1* mutant cells, an average of 4.0% and 17.3%, respectively (Fig. 6). On the other hand, wild-type

Figure 6. *stn1-13* mutants, as well as *cdc13-1* mutants, accumulate single-stranded DNA at telomeres. Cultures of YNG65 (wild-type), YNG32 (*cdc13-1*) and YNG429 (*stn1-13*) were grown to log phase at 25°C and transferred to 37°C for 5 hr. Native DNA and denatured DNA from these cultures were prepared as described in Materials and Methods. The percentage of single-stranded DNA indicated (for each sample, average and standard error from four independent experiments are shown) represents the amount of hybridization of native DNA to the DNA probe compared with the amount of hybridization measured in denatured DNA from the same sample. A Y' probe, corresponding to sequences in subtelomeric regions of chromosomes, was used. Comparison of *top left* and *bottom left* panels shows that *stn1-13* and *cdc13-1* cells accumulate

single-stranded DNA in subtelomeric regions but not in centromeric regions of the chromosomes. Probe *TRP1*, which hybridizes to unique sequences close to the centromere of chromosome IV, was used. For each panel, the upper row corresponds to native DNA and the lower row to denatured DNA, as indicated. It is important to note that the amount of denatured DNA deposited in each slot is only 1/10 of the amount of native DNA deposited. For each strain in *top* and *bottom left* panels, two concentrations of DNA (native or denatured) were deposited side by side, corresponding to 0.50 and 0.25 µg of DNA (from left to right). (*Top right*) In contrast to *stn1-13* and *cdc13-1* mutants, *cdc15-2* and *cdc16-1*, respectively, late M and mid-M phase mutants, all at 37°C for 5 hr, did not accumulate single-stranded DNA in subtelomeric regions. (*Bottom right*) The control with the same strains using the centromeric probe. For each strain in *top* and *bottom right* panels, 0.50 µg native DNA and 0.05 µg denatured DNA were deposited in each slot.

cells had practically no single-stranded subtelomeric DNA (average of 0.9 %). When blots were probed with a centromeric probe (*TRP1*), no dramatic changes in the amounts of single-stranded DNA were detected in *cdc13* and *stn1* mutant cells, as compared with control wild-type cells (Fig. 6).

To eliminate the possibility that single-stranded DNA accumulation was caused by cell cycle effects (wild-type cells continue to grow and cycle at 37°C, whereas *cdc13* and *stn1* cells arrest in G₂), we also prepared genomic DNA from *cdc15* (arrest in late M phase) and *cdc16* (arrest in mid-M phase) cells at 37°C. The amounts of single-stranded DNA found in arrested *cdc15* and *cdc16* cells were identical to those found in wild-type cells (Fig. 6).

Stn1 and *Cdc13* regulate the size of telomeres

Because the increased amount of single-stranded DNA in subtelomeric regions of *stn1-13* and *cdc13-1* mutants suggested a role for these two genes in telomere metabolism, we measured the size of telomeres following a loss of function of *STN1* or *CDC13* or overexpression of these genes. To do this, genomic DNA of various strains was digested with *XhoI*, which results in the release of chromosome extremities from a region located in the Y' subtelomeric regions [Louis and Borts 1995]. Digested DNAs were then detected on Southern blots by hybridization to radioactively labeled TG₁₋₃ sequences (see Materials and Methods).

The significant conclusion of these experiments is

that both *cdc13-1* and *stn1-13* mutants display elongated telomeres. As shown in Figure 7 (left), both *cdc13-1* cells grown at 29°C (maximal permissive temperature) for 40 generations and *stn1-13* cells grown at 37°C (temperature allowing slow growth attributable to the leakiness of the mutation) for 40 generations contained lengthened telomeres compared with an isogenic wild-type strain. Lengthening of telomeres in these mutant strains was clearly visible by the appearance of DNA fragments migrating at ~2 kb, corresponding to an average increase of 0.9 kb of telomeric repeats. Lengthening of telomeres appeared quantitatively similar in *cdc13-1* cells at 29°C and *stn1-13* cells at 37°C. Importantly, at 25°C *stn1-13* cells displayed telomeres elongated to the same extent as at 37°C [Fig. 7 (left), cf. lanes 5 and 6]. In the same vein, telomere size was also larger in *cdc13-1* cells at 25°C than in a wild-type congenic strain [Fig. 7, cf. lanes 2 and 3 (left) and lanes 2 and 3 (right)]. The *cdc13-1* strains used here and throughout this study were backcrossed five times against the wild-type genetic background used in our laboratories. Therefore, the fact that *cdc13-1* cells have longer telomeres than wild-type cells at permissive temperatures cannot be attributed to differences in telomere lengths in different genetic backgrounds. This was confirmed by the fact that *cdc13-1* cells transformed with YEp-CDC13 and grown at 25°C for 20 generations had shorter telomeres than *cdc13-1* cells at 25°C (Fig. 7, right). The fact that telomere size in *cdc13-1* YEp-CDC13 cells did not return to wild-type size [Fig. 7 (right), cf. lanes 2, 3, and 4] probably reflects the progressive nature of telomere size regulation. Assuming a telo-

Figure 7. *cdc13-1* and *stn1-13* mutant cells display an increase in telomere size. *Xho*I-digested DNA samples were hybridized to a TG₁₋₃ telomeric probe (see Materials and Methods). *Xho*I cuts in the Y' fragment of chromosomes (Louis and Borts 1995) at ~1.1 kb from the end in our genetic background. Most chromosomes have a Y' subtelomeric region and, therefore, the sizes of the fragments migrating around 1.1 kb are proportional to the sizes of telomeres. Some chromosomes do not have a Y' region (Louis and Borts 1995). From these chromosomes, *Xho*I cutting typically generates the fragments clearly seen on the gels around 1.9, 2.3, 3.2, and 4 kb (arrowheads in lane 1 of left panel). (Left, lane 1) MW markers; (lane 2) isogenic wild-type (YNG65); (lane 3) *cdc13-1* (YNG32) at 25°C; (lane 4) *cdc13-1* (YNG32), 40 generations at 29°C; (lane 5) *stn1-13* (YNG429) at 25°C; (lane 6) *stn1-13* (YNG429), 40 generations at 37°C; (lane 7) YEp-GAL-CDC13 (YNG510), 40 generations in galactose medium; (lane 8) YEp-GAL-STN1 (YNG509), 40 generations in galactose medium. The lower arrowhead of lane 1 marks the migration of *Xho*I-cut telomere fragments in wild-type cells, ~1.1 kb. The small elongation of telomeres in *cdc13-1* cells at 25°C is clearly visible in lane 2 not only from the increased size of the *Xho*I-cut telomeres (from 1.11 to 1.17 kb) but also from the increased sizes of the four upper bands (marked by arrowheads in wild-type cells) corresponding to the migration of telomere fragments that do not contain Y' sequences in their subtelomeric regions (see Louis and Borts 1995). (Right, lane 1) MW markers; (lane 2) isogenic wild-type (YNG65); (lane 3) *cdc13-1* (YNG32) at 25°C; (lane 4) *cdc13-1* transformed with YEp-CDC13 (YNG519), 20 generations at 25°C; (lane 5) MW markers; (lane 6) *cdc13-1* transformed with YEp-STN1 (YNG128) at 25°C; (lane 7) *cdc13-1* transformed with YEp-STN1 (YNG128), 40 generations at 29°C.

mere length of ~0.3 kb in wild-type cells, telomere length in *stn1-13* cells at 25°C or 37°C and in *cdc13-1* cells at 29°C was measured to be ~1.2 kb, yielding a 0.9-kb elongation, whereas elongation in *cdc13-1* cells at 25°C was around 0.06 kb.

Interestingly, overexpression of *STN1* in *cdc13-1* cells suppressed telomere elongation (Fig. 7, right). Therefore, in *cdc13-1* YEp-STN1 cells grown at 25°C for 40 generations, telomere size was identical to that in a wild-type congenic strain [Fig. 7 (right), cf. lanes 2 and 6], smaller than in *cdc13-1* cells (Fig. 7, right). At 29°C, the effect of *STN1* overexpression on telomere size in *cdc13-1* cells was dramatic, resulting in shortening by ~1 kb [Fig. 7, cf. lane 4 (left) and lane 7 (right)]. Telomeres in *cdc13-1* YEp-STN1 cells at 29°C were even slightly shorter than in a wild-type congenic strain [Fig. 7, cf. lanes 2 and 7 (right)] (see Discussion).

Strains containing the *stn1-13* mutation reached telomere size equilibrium, as the extent of elongation (~1 kb) was constant after several weeks of continuous culture in liquid medium at 25, 30, or 37°C (data not shown). In contrast, *cdc13-1* cells cultured for 2 weeks at 29°C continued to exhibit telomere elongation beyond the size shown in Figure 7 (40 generations). On Southern blots, *cdc13-1* telomere sizes under such conditions were difficult to measure (because of a very smeary signal) but appeared to be around 3.0 to 4.0 kb long (data not shown). Whether telomere length in *cdc13-1* cells eventually

reaches an equilibrium at 29°C remains to be determined.

On the other hand, in a wild-type background, neither overexpression of *CDC13* from a centromeric plasmid under the control of the *GAL1* promoter [Fig. 7 (left), lane 7], nor overexpression of *STN1* from an episomal plasmid under the control of the *GAL1* promoter [Fig. 7 (left), lane 8] had any detectable effect on telomere size.

These data strongly implicate both *STN1* and *CDC13* in telomere function and/or maintenance. Interestingly, however, the effects of these mutations on telomere length did not correlate quantitatively with their effects on the generation of subtelomeric single-stranded DNA noted above. Therefore, Figure 7, lanes 4 and 6 (left), which represent similar telomere sizes in *cdc13-1* at 29°C and *stn1-13* at 37°C, respectively, corresponded under the same growth conditions, to 17% and 4% of single-stranded DNA in subtelomeric regions, respectively, as seen above (subtelomeric single-stranded DNA in *stn1-13* was not measured at 25°C, but was 1.5% at 30°C).

Finally, to rule out the possibility that the higher than normal amounts of single-stranded DNA in these mutants might artifactually affect the migration of *Xho*I-digested fragments in the teloblot experiments, we performed additional controls in which the DNA was denatured before separation in agarose gels, as explained in Materials and Methods. Under such conditions, all of the

telomere lengthening effects of *stn1-13* and *cdc13-1* mutations were observed, as described in Figure 7 where the samples were denatured only after migration on agarose gels (data not shown).

Discussion

In this report, we describe *STN1*, an essential gene of *S. cerevisiae*, identified as a suppressor of the *cdc13-1* mutation. At their primary amino acid sequence level, neither Cdc13 (Garvik et al. 1995) nor Stn1 (present work) display homologies with proteins identified previously or even share consensus sequences with known motifs. The Stn1 protein is most probably required during S phase and failure to execute its function activates the *MEC3*- and *RAD9*-dependent G_2/M checkpoints, conferring cell cycle arrest. Based on two-hybrid analysis, Stn1 and Cdc13 interact physically and both proteins appear to be involved in the regulation of telomere length. We propose a model in which Cdc13 and Stn1 are part of an alternatively active and inactive telomerase complex (Fig. 8).

STN1 is a novel, essential, S-phase gene

STN1 can be considered a cell division cycle gene because a loss-of-function mutation arrests cells with a uniform phenotype while metabolic growth continues, a characteristic shared by all *cdc* mutants (Pringle and Hartwell 1981).

Three lines of evidence indicate that *STN1* function is required during S phase. First, *STN1* was isolated by virtue of its ability to rescue a deficiency in a function of *CDC13*, a gene implicated at the end of S phase (Garvik et al. 1995). Second, a loss of *STN1* function, either following Stn1 protein depletion in a *STN1* disruptant or in a *ts stn1* strain, activates the *RAD9* and *MEC3*-dependent G_2/M checkpoints (Weinert et al. 1994). Third, reciprocal shift experiments with cell cycle inhibitors indicate that *STN1* function is executed during late S phase or G_2 .

Stn1 and Cdc13 have a function in telomere maintenance

STN1 was isolated as a low-copy suppressor of the *cdc13-1* mutation at the semi-permissive temperature of 30°C, but is, in fact, a multicopy suppressor of that mutation at more restrictive temperatures. Absence of rescue of the *CDC13* defect at 37°C suggests that a minimal level of Cdc13 activity is required for an essential function, which cannot be bypassed by elevated levels of Stn1. This is compatible with the idea that Stn1 and Cdc13 function as a complex, a hypothesis supported by the findings that they physically associate based on two-hybrid analysis and that the *cdc13-1* and *stn1-13* mutations genetically interact (Fig. 2D).

In this study, we present evidence that loss of function of either *STN1* or *CDC13* produces a dramatic lengthening of telomeres, directly measured by hybridizing chro-

Figure 8. A model for regulation of telomerase by Stn1 and Cdc13. (Top) An active telomerase complex, formed of telomerase, Est1 and Cdc13. Cdc13 is shown bound to the single-strand G-rich extension present at the chromosome end, as proposed recently based on specific binding of Cdc13 to single-strand G-rich telomeric DNA and genetic interactions with Est1 (Nugent et al. 1996). Est1 binds to the free 3' terminus of the G-rich strand (Nugent et al. 1996) and to telomerase (Steiner et al. 1996). In this configuration, telomeres elongate. In response to a signal indicating the end of telomere elongation, when telomeres have reached a stasis (Rap1/Rif1 signaling pathway, e.g., Conrad et al. 1990; Lustig et al. 1990; Kyriou et al. 1992), Stn1 binds to the active telomerase complex (or becomes active if already bound to the complex in an inactive form), inducing conformational changes in Est1 and/or in Cdc13, which lose the ability to recruit telomerase. As a result, telomere elongation terminates (see text for further explanations). In this model, the depiction of Stn1 and telomerase as being reversibly, physically dissociated is speculative and it is equally possible that such a complex may be constitutive but reversibly active and inactive. Likewise, this schematic representation does not address the possibility of other proteins being part of the telomerase complex.

mosome ends with TG_{1-3} sequences. In addition, we have shown that *stn1-13* cells exhibited an increased amount of single-stranded DNA in subtelomeric regions of the chromosomes at restrictive temperature, as has been shown recently for *cdc13-1* mutants (Garvik et al. 1995; Lydall and Weinert 1995). The amount of single-stranded DNA found here in a *cdc13-1* strain at 37°C (average of 17%, vs. 0.95% in wild-type cells) was much higher than that reported by Garvik et al. (1995) under identical conditions (average of 5%). The discrepancy may be attributable to different genetic backgrounds used. In addition, the fact that *stn1-13* cells display a smaller amount of single-stranded DNA (average of 4%) than *cdc13-1* cells might be attributable to leakiness of the *stn1-13* mutation. Alternatively, the difference might reflect different functions of the two proteins. In both *stn1-13* and *cdc13-1* mutants, the increased percentage of single-stranded DNA in subtelomeric regions

and telomere lengthening do not seem to correlate tightly. In *stn1-13* cells for instance, the percentage of single-stranded DNA at the telomeres was 1.5% at 30°C and 4% at 37°C, whereas telomeres were elongated to the same extent at either temperature. It should also be noted that each parameter (single-stranded DNA and telomere size) was measured under a variety of conditions, particularly at different intervals following the shift to the semi-permissive temperature.

Both the Cdc13-1 and Stn1-13 mutant proteins conferred a defect in telomere maintenance. Lengthening of telomeres in *cdc13-1* cells at 25°C, ~0.06 kb, was modest compared with that (nearly 1 kb) in *stn1* mutants at 25°C or 37°C, or in *cdc13-1* mutants at 29°C after 40 generations of growth. It may be that in contrast to the Stn1-13 protein, which is defective at all temperatures, the Cdc13-1 protein displays a maximal defect only at elevated temperatures. However, it should be noted that the effect at permissive temperature, although modest, is significant because it was measured relative to a congenic strain. This was confirmed by reversal of telomere elongation in *cdc13-1* cells at 25°C following overexpression of CDC13. Moreover, telomere elongation in *cdc13-1* cells was reversed by overexpression of *STN1*. This result is consistent with the idea that Stn1 and Cdc13 form a codependent functional complex in vivo and that they participate in the same telomeric function. Both telomere elongation and the growth defect in *cdc13-1* cells at 29°C could be suppressed by overexpressing *STN1*. However, it remains to be determined whether or not these two phenotypes of *cdc13-1* at 29°C are related.

stn1-13 mutant cells growing continuously at 25°C displayed the same lengthening of telomeres as cells growing for 40 generations at 37°C, which strongly suggests a constant steady state length rather than a continuous lengthening of telomeres. This suggests that telomere size in *stn1-13* mutant cells attains an equilibrium reminiscent of the situation in *rap1^t* mutants (Kyrion et al. 1992). In contrast, *cdc13-1* cells grown at their maximal permissive temperature, 29°C, exhibited a continuous lengthening of telomeres. It is not known whether these cells eventually reach an equilibrium in telomere size or rather exhibit a continuous and progressive telomere lengthening. It is likely that an equilibrium in telomere size could be achieved independently of Stn1/Cdc13 function by alternative mechanisms of telomere length regulation, such as telomeric rapid deletion (Li and Lustig 1996). Increased telomere length is apparently not a lethal event because *stn1-13* cells remain viable at temperatures (permissive or restrictive for growth) at which telomere elongation is observed. In contrast, mutations that promote continuous shortening of telomeres, such as *est1* or *tlc1*, cause cell death (Lundblad and Szostak 1989; Singer and Gottschling 1994).

It has been speculated that Cdc13 might be involved in the repair of single-stranded telomeric damage or in the protection of telomeric ends from degradation (Garvik et al. 1995). One suggested function for Cdc13 might be to regulate the size of a short TG₁₋₃-containing telomeric

sequence, the TG₁₋₃ tail, which represents a normal intermediate in telomere replication (Garvik et al. 1995; Wellinger et al. 1996). Stn1 might form a complex with Cdc13 at the TG₁₋₃ tails, the function of which would be to regulate the access of telomerase (see below) and of the putative 5' → 3' exonuclease postulated by Wellinger et al. (1996) to have a role in TG₁₋₃ tail size regulation. According to this hypothesis, Stn1/Cdc13 might function as the putative "cap" protein proposed to regulate telomere length in yeast (McEachern and Blackburn 1995).

A model for Stn1 and Cdc13 function in telomere size control

In a recent study, the isolation of a second mutant allele of *CDC13*, *est1-4*, or *cdc13-2^{est}*, defective in telomere maintenance and therefore producing a progressive shortening of telomeres, was reported (Nugent et al. 1996). These authors also showed that Cdc13 specifically binds to single-strand G-rich telomeric DNA and that it genetically interacts with Est1. Because Est1 protein associates with telomerase (Lin and Zakian 1995; Steiner et al. 1996), a Cdc13–Est1 complex might form at the single-stranded telomeric overhang to regulate telomerase activity (Nugent et al. 1996). Contrary to *cdc13-2^{est}* cells, *cdc13-1* cells do not exhibit telomere shortening but, rather, display a modest telomere lengthening at 23°C (see Fig. 1A, lanes 14 and 15, in Nugent et al. 1996; this study). The Cdc13-1 mutant protein could be defective in protection of the telomere end against degradation and the Cdc13-2 mutant protein defective in telomerase loading. This is based on the existence of a synthetic phenotype between *Δtlc1* and *cdc13-1* and on the absence of a genetic interaction between *Δtlc1* and *cdc13-2^{est}*, as well as on the effect of *cdc13-2* and absence of an effect of *cdc13-1* on telomere size regulation (Nugent et al. 1996).

Because telomeres elongate in *stn1-13* and *cdc13-1* mutants, Stn1 and Cdc13 appear to act as negative regulators of telomerase activity. This is assuming that telomere elongation in these situations is attributable to telomerase-dependent events and not to recombination. On the other hand, the shortening of telomeres in *cdc13-2^{est}* mutants suggests that Cdc13 is a positive regulator of telomerase activity (Nugent et al. 1996). The fact that overexpression of *STN1* produces telomere shortening when Cdc13 is deregulated (in *cdc13-1* at 29°C) (Fig. 7), presumably by binding too much of Cdc13 and decreasing its telomerase-loading activity, is consistent with this interpretation. Furthermore, the facts that Stn1 and Cdc13 form a physical complex and that they genetically interact suggest that the inhibitory effect of Stn1 operates through Cdc13. The *cdc13-2* mutation specifically impairs binding between Cdc13-2 and Est1 (because overexpression of *EST1* rescues *cdc13-2*, but not *cdc13-1*, see Nugent et al. 1996). Consequently, telomerase access is compromised and telomeres shorten (Nugent et al. 1996). Alternatively, Cdc13-2 might recruit Stn1 more efficiently, therefore increasing inhibition of the telom-

erase complex. In the *cdc13-1* mutant, binding between Cdc13-1 and Stn1 is impaired (overexpression of *STN1* rescues *cdc13-1*, possibly by restoring wild-type levels of Stn1/Cdc13 complex). The telomerase complex consequently would remain in a predominantly active form and telomeres continue to elongate beyond normal size. In the *stn1-13* mutant, binding between Stn1-13 and Cdc13 would also be affected (Stn1-13 mutant protein is defective in some function related to Cdc13 because more Stn1-13 than Stn1 is required to rescue *cdc13-1*), but, presumably, to a lesser extent than binding between Stn1 and Cdc13-1. This also results in failure to inhibit telomerase and telomere lengthening. Stn1-13 is presumably partially active as an inhibitor of telomerase access, as a new equilibrium in telomere size is achieved readily (~0.9 kb longer than wild-type telomeres). Cdc13-1 is likely to be more defective in its capacity to recruit the putative telomerase inhibitor, Stn1, which is reflected by a failure of *cdc13-1* cells to rapidly attain a new equilibrium in telomere size at 29°C.

Based on these observations, we propose that Cdc13 is alternatively a positive and negative regulator of telomerase activity. We further propose that Stn1 is a negative regulator of telomerase activity through its interaction with Cdc13. According to this model, a putative telomerase complex would be composed of at least Tlc1, Est1, Stn1, and Cdc13. Cdc13 would bind to the G-rich strand of telomeric DNA and to Est1 (Fig. 8). Est1 would bind to the G-rich strand and to telomerase, therefore allowing telomeres to elongate. In response to a signal indicating sufficient telomere elongation, Stn1 would be recruited by Cdc13, therefore inhibiting the telomerase-loading activity of Cdc13 and/or Est1 (Fig. 8). It should be noted that Stn1 might be present constitutively in the putative telomerase complex but in an inactive form, becoming active as an inhibitor periodically.

Deregulation of telomere size observed in *stn1-13* and *cdc13-1* mutants might be similar to that observed in some *rap1* mutants (Conrad et al. 1990; Hardy et al. 1992; Kyrion et al. 1992; Moretti et al. 1994). In *ter1* mutants affected in the telomerase RNA, the severity of deregulation of telomere length control has been shown to be directly proportional to the loss of Rap1 binding to the mutated telomeric repeats (McEachern and Blackburn 1995; Krauskopf and Blackburn 1996). It is tempting to speculate that the Stn1/Cdc13 complex might act in cooperation with Rap1 in the control of telomerase accessibility to the TG₁₋₃ tails via elongation of the G-rich strand. This could be achieved either by association with the telomeric repeats at privileged sites or direct association with Rap1.

One can only speculate at the moment on the nature of the signals required for Stn1 and Cdc13 activities (Fig. 8). It should be noted that both genes are essential, whereas precise telomere size control is not essential and is not required at each cell cycle. The essential function of Cdc13 may reside in its role of protection of the chromosome ends, as the damage caused by the *cdc13-1* mutation at restrictive temperature is lethal, at least when the cell cycle cannot be halted via the *RAD9* sur-

veillance system (Garvik et al. 1995). Therefore, Stn1 and Cdc13 activities (Fig. 8) must be required at each cell cycle and are likely to be controlled by cell cycle regulatory proteins. In this context, Stn1 and/or Cdc13 might mark the completion of S phase and allow continuation of the cell cycle, a proposed function for TG₁₋₃ tails (Wellinger et al. 1993). Upstream regulatory signals of Stn1 and Cdc13 activities at the telomere other than cell cycle-dependent ones, might be signals controlled by the Rap1/Rif1 pathway (Conrad et al. 1990; Lustig et al. 1990; Kyrion et al. 1992).

Materials and methods

Strains, media, genetic procedures, and growth conditions

Yeast strains used in this study were derivatives of BF264-15Daub (*ade1 his2 leu2-3,112 trp1-1a ura3Dns*) (Richardson et al. 1989), except for the strains used in the two-hybrid system. The *cdc2*, *cdc9*, *cdc13*, and *cdc17* mutants were made isogenic by backcrossing several times with the parental wild-type strains (Table 1). All *cdc* strains as well as the *rad9::LEU2* strain were kindly provided by Leland Hartwell (University of Washington, Seattle, WA).

Yeast cultures were grown at the indicated temperatures in YEP (1% yeast extract, 2% Bacto-peptone, 0.005% adenine, 0.005% uracil) supplemented with 2% glucose (YEPD), sucrose or galactose, or in minimal medium (Sherman 1991). Induction of genes placed under the control of the *GAL1* promoter was done by transferring cells growing in sucrose- or glucose-containing medium into galactose-containing medium after several washes when in liquid culture or by streaking cells from glucose-based plates onto galactose-based plates. For repression of the *GAL1* promoter, cells growing in galactose-containing medium were also washed several times before transferring into glucose-containing medium.

Yeast genetic procedures were as described in Sherman et al. (1986). Gene disruption was performed as described by Rothstein (1983). Plasmids bearing the *GAL1* promoter were constructed by cloning the *GAL1* promoter into the *EcoRI-BamHI* sites of YCplac or YEplac plasmids (Gietz and Sugino 1988). All constructs were made by using PCR to adapt the relevant restriction sites to the sequence of the genes.

Cloning and characterization of STN1

cdc13-1 cells were transformed with a genomic library constructed in YCp50 (Rose et al. 1987) and plated onto uracil-lacking medium, at 25°C. Colonies were then replica-plated at 30°C and 37°C. Colonies that grew better than *cdc13* at 30°C but did not grow at 37°C were selected. These colonies were transferred onto medium containing 5-fluoro-orotic acid (5-FOA, from PCR Inc.). Two colonies were selected that could no longer grow faster than the *cdc13* strain at 30°C in the presence of 5-FOA. Plasmids recovered from these two colonies contained the same 7-kb insert. This insert was subcloned to find the smallest DNA fragment capable of rescue of *cdc13* at 30°C. A 2.2-kb *NruI/SacI* insert was selected, transferred in pBS-KS and sequenced using the sequenase kit (Amersham). An ORF of 1482 bases was found that potentially encoded a 494-amino-acid protein. No splicing consensus sequences were found. The sequence corresponding to the ORF plus 200 bp upstream (putative promoter) was amplified by PCR and, when cloned into single copy or multicopy plasmids, found to rescue *cdc13* at 30°C. Moreover, to ascertain that the complete gene had been

Table 1. Yeast strains

Strains	Relevant genotype	Source
NGY32	MATa <i>cdc 13-1</i>	this study
NGY65	MATa	S. Reed (Scripps Research Institute, La Jolla, CA)
NGY66	MATα	S. Reed (Scripps Research Institute, La Jolla, CA)
NGY104	MATa <i>rad9::LEU2</i>	this study
NGY112	MATa <i>cdc 13-1 rad9::LEU2</i>	this study
NGY123	MATa/MATα	this study
NGY128	MATa <i>cdc 13-1</i> pSTN1 (2μ, URA3)	this study
NGY154	MATa <i>cdc 13-1</i> pSTN1 (CEN, URA3)	this study
NGY156	MATa <i>cdc 13-1</i> pGAL::STN1 (CEN, URA3)	this study
NGY178	MATa <i>cdc 17-1</i>	this study
NGY194	MATa <i>cdc 13-1 mec3-2</i>	N. Grandin (Ecole Normale Supérieure, Lyon, France)
NGY203	MATa <i>cdc 9-8</i>	this study
NGY238	MATa <i>cdc 2-2</i>	this study
NGY291	MATa <i>cdc 9-8</i> pGAL::STN1 (2μ, URA3)	this study
NGY293	MATa <i>cdc 2-2</i> pGAL::STN1 (2μ, URA3)	this study
NGY295	MATa <i>cdc 13-1</i> pGAL::STN1 (2μ, URA3)	this study
NGY338	MATa/MATα STN1/ <i>stn1::TRP1</i>	this study
NGY346	MATa <i>stn1::TRP1</i> pGAL::STN1 (CEN, URA3)	this study
NGY356	MATa <i>mec 3-2</i>	N. Grandin (Ecole Normale Supérieure, Lyon, France)
NGY359	MATa <i>stn1::TRP1 rad9::LEU2</i> pGAL::STN1 (CEN, URA3)	this study
NGY391	MATa <i>stn1::TRP1 mec3</i> pGAL::STN1 (CEN, URA3)	this study
NGY396	MATa <i>stn1::TRP1</i> pGAL::STN1 (CEN, LEU2)	this study
NGY429	MATa <i>stn1::TRP1 pstn1-13</i> (CEN, LEU2)	this study
NGY447	MATa/MATα STN1/ <i>stn1::URA3</i>	this study
NGY451	MATa <i>stn1::TRP1 pstn1-13</i> (CEN, LEU2) pCDC13 (2μ, URA3)	this study
NGY452	MATa <i>stn1::TRP1 pstn1-13</i> (CEN, LEU2) <i>cdc 13</i>	this study
NGY467	MATa <i>stn1::TRP1 pstn1-13</i> (CEN, LEU2) <i>mec3</i>	this study
NGY497	MATa <i>cdc 14-1</i>	this study
NGY498	MATa <i>cdc 14-1</i> pGAL::STN1 (2μ, URA3)	this study
NGY499	MATa <i>cdc 15-2</i>	this study
NGY500	MATa <i>cdc 15-2</i> pGAL::STN1 (2μ, URA3)	this study
NGY506	MATa <i>cdc 17-1</i> pGAL::STN1 (2μ, URA3)	this study
NGY509	MATa pGAL::STN1 (2μ, URA3)	this study
NGY510	MATa pGAL::CDC13 (CEN, LEU2)	this study
MCY102	MATa/MATα pGAL4(1-147)-CDC13 pGAL4(768-881)-STN1	this study
MCY105	MATa/MATα pGAL4(768-881)-STN1 pGAL4(1-147)-p53 gene	this study
MCY106	MATa/MATα pGAL4(768-881)-STN1 pGAL4(1-147)-lamin gene	this study
MCY108	MATa/MATα pGAL4(768-881)-STN1 pGAL4(1-147)-CDC14	M. Charbonneau (Ecole Normale Supérieure, Lyon, France)

isolated, a cDNA library (gift from S. Elledge, Baylor College of Medicine, Houston, TX) was used to complement the *STN1* disruption (see below). A *stn1::TRP1* strain, kept alive with a *URA3* plasmid bearing wild-type *STN1* under the control of the *GAL1* promoter, was transformed with the cDNA library constructed in a *LEU2* plasmid (pACT1). Grown-up cells were cultured for several generations on leucine-lacking medium and then plated on 5-FOA-containing medium. The first colony that appeared was picked and the *LEU2* plasmid recovered. The insert was sequenced and the cDNA sequence found to encode the same ORF as the genomic *STN1* DNA, therefore confirming the absence of intronic sequences. The cloned *STN1* cDNA started 7 bp before the translation initiating ATG and ended 134 bp after the stop codon.

DNA sequences were analyzed by DNA Strider. Homology

searches were carried out using the BLAST program (Altschul et al. 1990) at NCBI, NIH.

STN1 disruption

The *STN1* entire ORF plus 200 bp upstream of the ATG was amplified by PCR and cloned into the *Bam*HI site of a pBS-KS plasmid deleted of its *Eco*RI site. The construct was then digested with *Nco*I and *Eco*RI, which released the first 840 bp of the gene and a 0.8-kb PCR fragment containing the *TRP1* gene was cloned between the *Nco*I site and the *Eco*RI site of *STN1*.

Another disruption was made using the *URA3* marker. The *STN1* ORF plus 200 bp upstream of the ATG was cloned at the *Bam*HI site of pBS-KS, cut with *Nco*I/*Afl*III, which released the

first 559 bp of the ORF, made blunt ended and religated to a 1.1-kb blunt ended *URA3*-containing fragment.

Both of these constructs were then cut with *Bam*HI in order to release the disrupted *STN1* gene that was then used to transform a diploid wild-type strain. Both the *stn1::TRP1/STN1* strain and the *stn1::URA3/STN1* strain were induced to sporulate and tetrads dissected. In each disruption, only two viable spores per tetrad were recovered and none of these spores were found to be Trp⁺ in the case of the *stn1::TRP1* disruption (52 tetrads dissected), or Ura⁺ in the case of the *stn1::URA3* disruption (30 tetrads dissected), indicating the disruption of an essential gene. The two diploid strains were then transformed with a centromeric plasmid containing the *LEU2* marker and *STN1* gene under the control of its own promoter. The strains were again induced to sporulate and tetrads dissected. This time, Trp⁺ spores were recovered from the *stn1::TRP1/STN1* diploid, which were also all Leu⁺, and Ura⁺ spores were recovered from the *stn1::URA3/STN1* strain, which were also all Leu⁺. This suggested that the disrupted gene was *STN1*.

Genomic DNA of the two diploid strains, *stn1::TRP1/STN1* and *stn1::URA3/STN1*, was prepared and disruption at the *STN1* locus was checked by PCR (for both *stn1::TRP1* and *stn1::URA3*) and Southern analysis (for *stn1::TRP1*).

Construction of a temperature-sensitive allele of *STN1*

The following oligonucleotides were used to amplify the entire *STN1* ORF plus 300 bp upstream of the ATG and 134 bp downstream of the stop codon: 5'-CCGGTACCCGCAACTAGT-CACG-3' and 5'-CCGGATCCTAACGTCCTTATTG-3'. Amplification was performed on a plasmid bearing an *STN1*-containing chromosomal fragment, under the following mutagenic conditions: 1 mM dCTP, 1 mM dTTP, 0.2 mM dGTP, 0.2 mM dATP, 3 mM MgCl₂, 0.5 mM MnCl₂, in standard PCR buffer (GIBCO BRL) and using standard *Taq* polymerase (GIBCO-BRL, ref. 18038067). Following a 30-cycle amplification, the PCR product was cleaned and 1/25 was used as a substrate for another round of amplification under the same mutagenic conditions. According to the gap repair method, the PCR product was cleaned and used directly to transform an *stn1::TRP1* YCp-GAL-*STN1*-*URA3* strain, together with a centromeric *LEU2* plasmid [YCp111] made linear by digestion and carrying *STN1* flanking regions at each extremity: region -300 to -2 (adenine of the ATG=position 0) at one end, and region +1528 to +2784 (thymine of the TAA stop codon is at position 1484 and poly (A) tail stops at position 1620) at the other end, making 299 bp upstream of *STN1* and 92 bp downstream of *STN1* available for homologous recombination with the *STN1* PCR fragment.

Cells were plated onto leucine-lacking glucose-containing medium at 25°C. Around 6,000 transformants grew up which were transferred to leucine-lacking glucose-containing liquid medium for 2 days and then plated on 5-FOA-containing medium at 25°C in order to force the loss of the YCp-GAL-*STN1*-*URA3* plasmid. Growing colonies were then replica-plated on YEPD medium at 37°C in order to screen for temperature-sensitive transformants.

One temperature-sensitive transformant was selected. The *LEU2* plasmid carrying the mutated allele of *STN1* was recovered from this strain and used to transform the *stn1::TRP1* YCp-GAL-*STN1*-*URA3* strain. After loss of the *URA3* plasmid on 5-FOA, the cells, which were kept alive now only by the presence of the *LEU2* plasmid, were found to be temperature-sensitive, indicating that the temperature-sensitive phenotype was linked to the mutated allele of *STN1* carried by the *LEU2* plasmid. This allele was called *stn1-13* and the plasmid carrying it, p*stn1-13*. As expected, *stn1::TRP1* p*stn1-13* cells transformed

with a centromeric plasmid carrying wild-type *STN1* lost their temperature-sensitive phenotype.

Block and release experiments

Cells were grown in YEPD medium, at 25°C overnight, then diluted to have a logarithmically growing population and grown for two more generations before hydroxyurea, 0.4 M, or nocodazole, 15 µg/ml, final concentrations, was added. Cells were treated for 2 hr with hydroxyurea or nocodazole at 25°C, then transferred for 1 hr, still in the presence of the drug, at 37°C or 30°C. Cells were then washed by centrifugation and released at either 37°C or 30°C and samples were fixed at 15-min intervals in 3.7% formaldehyde. Percentages of unbudded cells and of cells with small, medium or large buds were determined microscopically.

Immunofluorescence

Experiments of immunostaining of tubulin of mitotic spindles (using rat anti-tubulin, clone YOL1/34, from Sera-Lab) and simultaneous staining of nuclei with DAPI were conducted exactly as described in Pringle et al. (1991).

Two-hybrid experiments

The two-hybrid "kit" was kindly provided by Stephen J. Elledge. Experiments of protein-protein interactions using the two-hybrid system were performed as described previously (Fields and Song 1989; Durfee et al. 1993; Harper et al. 1993). *STN1* ORF was cloned in-frame with the *GAL4* activation domain (nucleotides 764–885) of pACT2 (pACT2 and pAS2 vectors have been described in Durfee et al. 1993) using an endogenous *Nco*I site at the 5' end and a PCR-adapted *Bam*HI site at the 3' end. *CDC13* ORF was cloned in-frame with the *GAL4* DNA-binding domain (nucleotides 1–147) in pAS2 using two PCR-adapted restriction sites—an *Nco*I site at the 5' end and a *Sma*I site at the 3' end of the ORF. Both constructs were transformed into the Y190 (MATa *gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 URA3::GAL* → *lacZ*, *LYS2::GAL(UAS)* → *HIS3* *cyh*^r) and Y187 (MATa *gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 met⁻ URA3::GAL* → *lacZ*) strains. The two strains Y190 pACT2-*STN1* and Y190 pAS2-*CDC13* were tested for β-galactosidase activity and found to be negative. Strains were then induced to mate, generating the Y190/Y187 pACT2-*STN1*/pAS2-*CDC13* diploid strain that was also tested for β-galactosidase activity, and found to be positive. β-Galactosidase activity was tested as described previously (Durfee et al. 1993; Harper et al. 1993) using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, from Sigma). Quantitation of β-galactosidase activity in strains expressing the different constructs was performed using the ONPG (2-nitrophenyl-β-D-galactopyranoside, from Boehringer) protocol (Rose and Botstein 1983).

Detection of single-stranded DNA

Genomic DNA was prepared as described for the preparation of DNA for 2D gel analysis of replication intermediates (Wu and Gilbert 1995). Fifty milliliters of cells were grown at 25°C and transferred, when in log phase of growth, to 37°C for 5 hr. Cells were then washed and resuspended in nuclear isolation buffer (NIB, 17% glycerol, 50 mM MOPS, 150 mM potassium acetate, 2 mM MgCl₂, 500 mM spermidine, 150 mM spermine, at pH 7.2). Cells were broken by addition of 425–600 µm-diameter glass beads and vigorous vortexing. Broken cells were transferred to a new tube, pelleted at 6500g at 4°C for 10 min and the pellet

resuspended in G₂ Qiagen lysis buffer containing 200 µg/ml ribonuclease A (Sigma). The lysate was incubated at 37°C for 30 min. Twenty microliters of proteinase K at 20 mg/ml was then added and the lysate incubated at 37°C for 60 min more. The lysate was then centrifuged at 6500g at 4°C for 10 min and the supernatant loaded on a pre-equilibrated 20/G Qiagen column, and allowed to pass through by gravity flow. The column was washed, DNA eluted, precipitated with isopropanol and resuspended in TE (10 mM Tris-HCl, 1 mM EDTA at pH 8.0). We routinely obtained 40 µl of genomic DNA at a concentration of 0.5 µg/µl.

Slot blot analyses were then performed as described in Garvik et al. (1995). Briefly, for each slot, 1.00, 0.50, or 0.25 µg of native DNA was adjusted to 165 µl with water. Fifteen microliters were removed and denatured at 65°C for 30 min in 0.2 M NaOH. Native and denatured DNA were then adjusted to 300 µl with 10 × SCP (1 M NaCl, 0.3 M Na₂HPO₄, 0.02 M EDTA at pH 6.8) and drawn through a Hybond N membrane (Amersham) using a GIBCO BRL Hybri-Slot manifold. DNA was linked to the membrane by UV exposure and membrane was pre-hybridized and then hybridized in 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 7% SDS, 500 µg/ml salmon sperm DNA. Y' and TRP1 DNA probes were made using [α-³²P]dCTP 3000 Ci/mM (Redivue, Amersham) using Appligene nona primer kit. Hybridization was quantified with a Storm Molecular Dynamics Phosphorimager.

Measurements of telomere lengths on teloblots

cdc13-1 and *stn1-13* cells were grown in liquid cultures for ~40 generations at the maximum temperature permitting growth (29°C for *cdc13-1*, 37°C for *stn1-13*). Wild-type cells bearing the *CDC13* and *STN1* genes on YCp-GAL plasmids were grown for 40 generations in galactose-based liquid medium. Genomic DNA was then prepared from these strains as described in Philippsen et al. (1991). DNA was then digested with *Xho*I to cut within the Y' regions of chromosomes (Louis and Borts 1995) and DNA fragments separated in a 0.9% agarose gel (in TBE) run in TBE buffer overnight. After denaturation, DNA was transferred onto nitrocellulose membrane and immobilized by baking at 80°C for 1 hr, under vacuum (Ausubel et al. 1995). The membrane was then pre-hybridized in 6 × SSC, 0.1% SDS, 1% nonfat milk and hybridized with a 270bp TG₁₋₃ ³²P-labeled probe. Control experiments in which DNA was denatured before separation of DNA fragments on agarose gels were performed to ensure that the higher percentage of single-stranded DNA in *stn1* and *cdc13* cells versus that in wild-type cells was not affecting migration of the telomeres. In this case, DNA fragments after *Xho*I cutting were separated under denaturing conditions using a 1% agarose gel (in H₂O) run in 40 mM NaOH, 2 mM EDTA.

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Stn1, a new *Saccharomyces cerevisiae* protein, is implicated in telomere size regulation in association with Cdc13.

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