In bakers' yeast, *in vivo* telomerase activity requires a ribonucleoprotein (RNP) complex with at least four associated proteins (Est2p, Est1p, Est3p, and Cdc13p) and one RNA species (Tlc1). The function of telomerase in maintaining chromosome ends, called telomeres, is tightly regulated and linked to the cell cycle. However, the mechanisms that regulate the expression of individual components of telomerase are poorly understood. Here we report that yeast RNase III (Rnt1p), a double-stranded RNA-specific endoribonuclease, regulates the expression of telomerase subunits and is required for maintaining normal telomere length. Deletion or inactivation of *RNT1* induced the expression of Est1, Est2, Est3, and Tlc1 RNAs and increased telomerase activity, leading to elongation of telomeric repeat tracts. *In silico* analysis of the different RNAs coding for the telomerase subunits revealed a canonical Rnt1p cleavage site near the 3′ end of Est1 mRNA. This predicted structure was cleaved by Rnt1p and its disruption abolished cleavage *in vitro*. Mutation of the Rnt1p cleavage signal *in vivo* impaired the cell cycle-dependent degradation of Est1 mRNA without affecting its steady-state level. These results reveal a new mechanism that influences telomeres length by controlling the expression of the telomerase subunits.

The ends of eukaryotic chromosomes are capped with special structures made of tandem DNA repeats and associated proteins, called telomeres. These structures protect chromosomes from end-to-end fusion, recombination, and nucleolytic degradation (1, 2). However, because the conventional DNA replication machinery cannot fully duplicate the ends of linear chromosomes, telomeric DNA will shorten with each round of replication, leading to a replicative senescence (3). To solve this end replication problem, most eukaryotes use the activity of an enzyme called telomerase to ensure the maintenance of telomeric DNA (4). Telomerase is a ribonucleoprotein reverse transcriptase that can extend the 3′ end of chromosomes using its RNA subunit as a template for the addition of telomeric repeats.

*In vitro*, telomerase activity requires a reverse transcriptase (Tert, Est2p in yeast) and an RNA template (Terc, Tlc1 in yeast). (5, 6). *In vivo*, yeast telomerase requires additional factors for function, including Est1p, Est3p, and Cdc13p (3, 7). Deleting any one of the *EST1*, *EST2*, *EST3*, or *TLC1* genes or a particular allele of the *CDC13* gene (*cdc13-2*) causes progressive telomere shortening leading to cellular senescence. Increased expression of individual telomerase genes may also lead to telomeric phenotypes. For example, overexpression of Tlc1 causes telomere shortening by sequestering the Tlc1-binding factor Ku (8, 9). Similarly, overexpression of Est1p may cause a slight telomere lengthening in some strains (10). On the other hand, induction of Est1, Est2, and Est3 mRNA expression in cells with defective nonsense-mediated mRNA decay decreases telomere length (11). These observations suggest that a variation in the expression levels of the different components of telomerase holonzyme influences telomerase function. However, the cellular mechanisms that regulate and balance the expression of the different components of the telomerase ribonucleoprotein remain unknown.

Telomere elongation occurs in late S or early G2/M phases of the cell cycle (12, 13). There is evidence that the catalytic core of telomerase (Est2p and Tlc1) is present at yeast telomeres throughout the cell cycle but requires an association of Est1p, which accumulates in the S phase of the cell cycle (14, 15). Telomerase activity on the telomeres is negatively regulated by Pif1p, a helicase that is thought to remove telomerase from the chromosome ends (16). In addition, the Rif proteins also exert a negative role on telomere length maintenance (17), and several other proteins involved in the DNA damage checkpoint and repair pathways were shown to influence steady-state telomere length *in vivo* (18). At least some of the factors regulating telomere length influence the activity of the telomerase subunits, yet very little is known about the mechanisms regulating the expression of the telomerase subunits.

Here we show that the expression level of several telomerase subunits is regulated post-transcriptionally by yeast RNase III (Rnt1p). Rnt1p is a double-stranded RNA-specific endoribonuclease involved in the maturation of many RNAs, including small nuclear RNAs, snoRNAs, and pre-rRNAs (19–21). Recently it was shown that Rnt1p regulates the expression of glucose-dependent genes, degrades unspliced mRNAs, and cleaves mRNAs with abnormal 3′ ends (22–25). The majority of Rnt1p substrates include a short RNA stem capped with an AGNN tetraloop structure (26). These particular structures are cleaved at a fixed distance from the tetraloop (27–29). Deletion of *RNT1* induced the expression of many components of telomerase.
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erase, leading to an increase in telomerase activity and elongation of the telomeres. A point mutation that impairs catalytic activity of Rnt1p without affecting its expression or interaction with RNA fails to restore normal expression of telomerase subunits or reduce telomere lengths. In silico analysis revealed a canonical Rnt1p cleavage site within the coding sequence of the Est1 mRNA. In vitro analyses showed that this predicted structure was directly cleaved by Rnt1p in the absence of other factors. Disruption of this Rnt1p cleavage site by silent mutations impaired the cell cycle-dependent regulation of Est1 mRNA, suggesting that Rnt1p triggers RNA cleavage in a cell cycle-dependent manner. These results demonstrate that the expression level of the telomerase subunits influences their function and reveal a new regulatory mechanism that controls the accumulation of telomerase-related RNAs.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—All yeast strains were grown and manipulated using standard procedures (30, 31). Strains W303-1A, rnt1Δ, rnt1-ts, and rnt1-D247/R were described earlier (22, 32–34). RNT1 (NLHY115) and rnt1Δ (NLHY12) cells harboring protein A-tagged EST2 were created by crossing strains YKF103 (MATα; ura3–52; ade2–101; lys2–801; leu2Δ1; trp1Δ1; his3Δ200/CF+ (TRP1 SLIP11)) (35) and DUY746 (MATα; leu2–3,112; his3; trp1; pep7; prc1; HIS3) (19). Strain SJ150 (EST1Δ–4) was constructed by inserting 4 silent mutations (T2061/C; G2064/A; C2067/T; C2070/T) within the EST1 sequence of BY4705, a derivative of BY4705. The DNA fragment used for the replacement of 4 nucleotides was created by cloning two PCR fragments in the pVZ1 vector (36). The first PCR fragment was amplified using primers 5′-CCAGACGAAGACATGA-GAGATATTAACGGTCCAAGTGC-3′ and 5′-GTGGTACCCGCGCCCCCTCGAGGAGTCG-3′. The amplification sequence corresponds to the 3′ end of EST1 and contains 4 mutations disrupting the Rnt1p cleavage site. The second PCR fragment was amplified using primers 5′-CAGATTGTACT-GAGAGTCCACC-3′ and 5′-GTATTGACAGATATATATATA-TTTGCCTGTCTTTGACAATTTTATATGCAAAATTAATAATAT-TATGTATTTCAGCAGCCTGCGGTATTTCAACC. The amplified fragment contains the URA3 gene and a short sequence of EST1. The integration of the DNA fragment in the yeast genome was selected by growth in the absence of uracil and the marker was removed by counterselection on 5-fluoroorotic acid. SLY178 (EST1Δ–4; bar1Δ::URA3) was generated by deleting the BAR1 gene from SLY150 as previously described (37). MLY30 strain (bar1Δ::HIS3) was constructed by deleting the BAR1 gene in BY4705a strain (MATα; ade2Δ::hisG; met15Δ0; trp1Δ63; lys2Δ0; ura3Δ0; his3Δ200; leu2Δ0; bar1Δ::HIS3) (38).

In Vitro RNA Cleavage—In vitro cleavage reactions were performed as described previously (29). Cleavage of model RNA substrates was performed using T7-synthesized transcripts and recombinant Rnt1p (0.2 pmol) (39) in a 20-μl reaction buffer containing 150 mM KCl and incubated 20 min at 30 °C. Cleavage of total RNA extracted from yeast was performed as described previously (22). Briefly, 50 μg of total RNA was incubated with 4 pmol of recombinant Rnt1p for 10 min at 30 °C as described for the cleavage of model substrates.

Analysis of mRNA Stability—The stability of the different mRNAs was determined after inhibiting transcription using 10 μg/ml of thiolutin as previously described (22). Cells were grown at 26 °C to mid-log phase and RNA was extracted at different time points after the addition of the drug. The levels of mRNA were quantified by real-time PCR using RPR1 (pol III transcript) as an internal control. Half-lives were estimated using the single exponential decay formula: t½ = 0.693/λ. Thiolutin was kindly provided by Pfizer (Groton, CT).

Primer Extension and Northern Blot Analysis—Primer extension was performed using a primer specific for EST1 (5′-GGGTTGACGACGACCCGCTG-3′) as described previously (19). Extended products were separated on a 6% denaturing polyacrylamide gel and visualized by autoradiography. Northern blots were performed as described previously (19) using 15 μg of total RNA and a 1% denaturing agarose gel or a 4% polyacrylamide gel. The RNA was visualized by autoradiography using randomly labeled probes corresponding to specific genes. The RNA was quantified using Instant Imager (PerkinElmer Life Sciences).

Real-time PCR Analysis—Synthesis of cDNA was performed using 2 μg of total RNA and SuperScript II Reverse Transcriptase (Invitrogen). Gene-specific primer protocol using a “Flap sequence” common to all primers was used to avoid DNA amplification during subsequent PCR amplification. The cDNA was purified with Wizard SV Gel and PCR Clean-Up System (Promega). Q-PCR was performed on an ABI PRISM 7700 (Applied Biosystems) as previously described (40). PCR were done in triplicate using a 25-μl reaction volume. TaqMan probes (5′-6FAM, 3′-TAMRA) and specific primers (Integrated DNA Technologies, Coralville, IA) were used at concentrations of 250 and 900 nM, respectively, in a reaction buffer containing 50 mM KCl, 5.5 mM MgCl2, 10 mM Tris, pH 8.0, with 0.625 units of homemade Tag (41), 0.25 unit of UDQ (New England Biolabs, Pickering, ON), 50 nM ROX Reference Dye (Invitrogen), 200 μM dATP/dGTP/dCTP, 400 μM dUTP (Amersham Biosciences). Data were analyzed by the comparative threshold method. All primers and probes used for real-time PCR are listed in Table 1.

Telomere Length Analysis—Telomere length was analyzed as described earlier (42). Genomic DNA was extracted from cells grown to mid-log phase. 5 μg of genomic DNA was digested with XhoI, separated on a 1% agarose gel, and transferred to a nylon membrane (Hybond N+, Amersham Biosciences). DNA was visualized by autoradiography with a randomly labeled telomeric probe.

Cell Cycle Synchronization and FACS Analysis—Cells were synchronized in G1 by the addition of α-factor (Bioshop, Burlington, ON) at a final concentration of 0.5 μg/ml for 3 h. Cells were harvested and resuspended in fresh media containing 200 μg/ml Pronase (Roche Diagnostics) to release the cells from their arrest. Samples were taken at different time points to either extract RNA or monitor the release into the cell cycle by FACS analysis as described previously (34). The RNAs corre-

* D. Ursic, personal communication.
sponding to the different genes was quantified by real-time PCR using Rpr1 as an internal control.

**Western Blot and Telomerase Activity**—Telomerase assays were performed as previously described (35). The EST2 gene was modified to incorporate an N-terminal Protein A tag as described (35). Immunoprecipitation was performed using IgG-Sepharose beads (Amersham Biosciences) and 2 mg of total protein extracted from either H9004 cells and two independent experiments for EST1–4 or RNT1Δ (NLYH12) cells. Extension of a telomeric primer (5’-TAGGG-TAGTAGTGGG-3’) was monitored to determine telomerase activity. Quantifications were performed using a Storm PhosphorImager (Storm 860, Amersham Biosciences). Western blots were performed using 1:7500 diluted (total protein) or 1:5000 diluted (immunoprecipitated protein) polyclonal rabbit anti-protein A antibodies (Sigma). Donkey anti-rabbit IgG conjugated with horseradish peroxidase was used as a secondary antibody to allow detection with ECL Plus reagents (Amersham Biosciences). Western blotting, stripping, blotting were incubated with mouse anti-PGK Ig diluted 1:500 (Molecular Probes) as primary and sheep anti-mouse IgG conjugated with horseradish peroxidase diluted 1:5000 (Amersham Biosciences) as secondary antibody. Detection and quantification were done using a Storm Fluorescence Imager (Storm 860, Amersham Biosciences).

**Non-linear Regression Analysis and Graph Generation**—The graphs shown in Fig. 5C were generated using GraphPad Prism version 4.03 for Windows (GraphPad Software, Inc., San Diego, CA). The data from three independent experiments for EST1–4; bar1Δ cells and two independent experiments for EST1; bar1Δ were used for the analysis. To choose the best equation to model the data, we compared the different classical equations and all polynomial order equations available in GraphPad. To select the best equation type, we used an F test with $p < 0.05$. Two-by-two comparisons were performed to identify the simplest model that best fit the data. The equation used is a polynomial of the fourth order ($Y = A + B \cdot X + C \cdot X^2 + D \cdot X^3 + E \cdot X^4$). The obtained values are shown in supplemental Table 1. The use of non-linear regression was required because the mRNA level is affected by many variables: mRNA decay, Rnt1p cleavage, constitutive expression, and cell cycle-dependent expression. The use of a non-linear regression also allows for the two main sources of variation in the measurement (normal experimental variation and variation in the cell synchronization) to be determined.

**RESULTS**

We previously performed a microarray analysis of RNA extracted from cells lacking RNT1 and compared it with RNA from wild type cells (22). After detailed inspection of the data, we noticed that many telomerase-related mRNAs were induced upon the deletion of RNT1 (Fig. 1A). This induced expression of RNAs coding for telomerase subunits was similar to that of known Rnt1p substrates like Rps22B (23) and Mig2 (22) mRNAs. The impact of RNT1 deletion on the expression of telomerase-related RNAs was verified by Northern blot analysis. Consistent with the microarray data, the mRNAs coding for the main components of the telomerase RNP complex (Est1, Est2, and Est3) and the telomerase RNA Tlc1 accumulated in the absence of Rnt1p (Fig. 1B). No major increase in the RNA amounts coding for other telomere-related proteins like Cdc13, Ten1, and Tel1 were detected by Northern blot (data not shown). We conclude that Rnt1p is required for normal expression of most core telomerase subunits.

If Rnt1p influences the stability of Est1, Est2, Est3, and Tlc1 RNAs, we expected the deletion of RNT1 to slow the turnover and increase the half-life of these RNAs. To examine this possibility, the half-life of each affected RNA was determined in wild type and rnt1Δ cells after inhibiting new transcription using thiolutin. Quantitative PCR analysis was used to estimate the mRNA amounts at different time points after transcription inhibition and the half-life was calculated for each RNA species (Table 2). We used two unrelated RNAs (Spt15 and Act1) with different half-lives and that are thought not to be directly affected by Rnt1p as controls (supplemental Fig. 1). Indeed, the

### Table 1

| Name            | Sequence                                      |
|-----------------|-----------------------------------------------|
| Primer EST1 For RTP | CTGCATTAAGATTACATTCACATTCAAGTTA               |
| Probe EST1 RTP   | GATGAAATTAGTGCGCGGCTCCGGAGTTTCAAACATAGATCGAAA |
| Primer EST2 For RTP | TCCCATTTGGAGCCCGAA ACTTT               |
| Primer EST2 Rev RTP | AGGACACCA ACTGAGAAGTTA            |
| Primer EST3 Rev RTP | GATGAAATTAGTGCGCGGCTCCGCCTATTTGACATATTGTA |
| Primer EST3 RTP   | ACCGGATATTGGCCCGAACATTT               |
| Primer EST3 For RTP | AACAGATTGCGCACTGAGAAGTTA            |
| Primer EST4 Rev RTP | GTGCTCATAGTCCGCTAACTGCGTACGTCTGGCTCAGGTACAG |
| Primer EST4 RTP   | TAGCTTGATCGGATCGGTACGTCTGGACGTGCAGG |
| Primer SPT1 For RTP | ACGCTGTTCCAAATTTACGGCTGGTTT               |
| Probe FLAP       | TATACGAGAATTACGCGAAACAG |
| Primer SPT1 Rev RTP | GATGAAATTAGTGCGCGGCTCCGCCTATTTGACATATTGTA |
| Primer SPT1 RTP   | ACCGGATATTGGCCCGAACATTT               |
| Primer SPT1 For RTP | AACAGATTGCGCACTGAGAAGTTA            |
| Primer SPT1 Rev RTP | GTGCTCATAGTCCGCTAACTGCGTACGTCTGGCTCAGGTACAG |
| Probe FLAP       | TATACGAGAATTACGCGAAACAG |
| Primer SPT1 RTP   | ACCGGATATTGGCCCGAACATTT               |
| Primer SPT1 For RTP | AACAGATTGCGCACTGAGAAGTTA            |
| Primer SPT1 Rev RTP | GTGCTCATAGTCCGCTAACTGCGTACGTCTGGCTCAGGTACAG |
| Probe FLAP       | TATACGAGAATTACGCGAAACAG |
| Primer SPT1 RTP   | ACCGGATATTGGCCCGAACATTT               |
| Primer SPT1 For RTP | AACAGATTGCGCACTGAGAAGTTA            |
| Primer SPT1 Rev RTP | GTGCTCATAGTCCGCTAACTGCGTACGTCTGGCTCAGGTACAG |
| Probe FLAP       | TATACGAGAATTACGCGAAACAG |
| Primer SPT1 RTP   | ACCGGATATTGGCCCGAACATTT               |
| Primer SPT1 For RTP | AACAGATTGCGCACTGAGAAGTTA            |
| Primer SPT1 Rev RTP | GTGCTCATAGTCCGCTAACTGCGTACGTCTGGCTCAGGTACAG |
| Probe FLAP       | TATACGAGAATTACGCGAAACAG |
| Primer SPT1 RTP   | ACCGGATATTGGCCCGAACATTT               |
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![Graph](Image)

**FIGURE 1. Deletion of RNT1 causes an increase in the steady-state level of RNAs of telomerase subunits.** A, illustration of the expression profiles of telomerase-related mRNAs upon the deletion of RNT1. The data were extracted from a previously performed microarray analysis using RNA extracted from wild type or rnt1Δ cells (22). Mig2 and Rps22B mRNAs are shown as positive controls, whereas Act1 mRNA is shown as a negative control. B, Northern blot analysis of RNAs coding for the telomerase RNP complex. Total RNA was extracted from wild type or rnt1Δ cells and visualized using gene specific randomly labeled probes. The RNA expression level was quantified using Instant Immager and the relative mRNA amount (RMA) was calculated using Act1 mRNA as reference. The RNA indicated below each gel is an average of three experiments with a S.D. ≤ 0.2 or less. C, graphical representation of the relative increases in the half-lives of the telomerase mRNAs upon the deletion of RNT1. The relative increase in the half-lives of the telomerase mRNAs was normalized to the half-life of Spt15 mRNA, which serves as a control for possible indirect effects caused by the slow metabolism of rnt1Δ cells. The different half-life values used to calculate the relative increase are indicated in Table 2. The data were obtained from three independent transcription inhibition experiments. D, Western blot analysis of Est2p expression. Total proteins were extracted from wild type or rnt1Δ cells expressing proA-EST2. Immunoprecipitation was performed using IgG-Sepharose beads. Proteins from total extract (upper panel) or from immunoprecipitation (lower panel) were separated using SDS-PAGE and Est2p was visualized using antibodies against the protein A. The expression level of Est2p was normalized to the IgG heavy chains or to Pgk1p. The relative protein amount (RPA) is an average of three experiments with a S.D. ≤ 0.6.

**TABLE 2**

| Gene Name | Strain | RNT1 | rnt1Δ |
|-----------|--------|------|-------|
| EST1      |        | 10.8±0.6 | 31.2±5.5 |
| EST2      |        | 7.2±1.2  | 22.1±6.2  |
| EST3      |        | 17.9±3.6 | 34.5±5.7  |
| TLC1      |        | >60.0    | >60.0     |
| SPT15     |        | 12.4±2.3 | 18.7±3.8  |

Half-lives of these control RNAs were only slightly longer in rnt1Δ cells when compared with that in wild type cells due to a general slow metabolism of rnt1Δ cells (Table 2 and data not shown). In contrast, these experiments showed a significant increase in the half-lives of Est1, Est2, and Est3 mRNAs (Table 2). As previously described, the Tlc1 RNA was already very stable in wild type cells (43), and no decay was observed in both wild type and rnt1Δ cells even after 1 h of transcription inhibition (Table 2). When normalized to Spt15, the relative half-lives of Est1 and Est2 were about 2 times longer in rnt1Δ than in wild type cells, whereas that of Est3 was only slightly longer in rnt1Δ cells (Fig. 1C). If the increase in the half-life of the Est2 mRNA of about 2-fold was significant, it should lead to a corresponding increase in the amount of Est2p. Indeed, deleting RNT1 in cells expressing a protein A-tagged version of the telomerase catalytic subunit Est2p increased the expression of both mRNA and protein expression by 2 to 3 times as compared with that detected in wild type cells (supplemental Fig. 2 and Fig. 1D). We conclude that deletion of RNT1 slows the decay rate of several telomerase-related mRNAs leading to an increase in the expression of these telomerase subunits.

To evaluate the impact of Rnt1p on telomerase function, we examined telomere length in wild type and rnt1Δ cells by Southern blotting. DNA was extracted from relevant strains and digested with the restriction enzyme XhoI. Due to a conserved XhoI site in the telomere-proximal Y′-elements, many terminal restriction fragments appear as somewhat smeary bands of about 1.2 kb size on such blots (44). In the absence of Rnt1p, the size of the terminal restriction fragments increased as compared with that of wild type cells, indicating that the deletion of RNT1 increases telomere length (Fig. 2A). This increase in telomere length is directly linked to the expression of Rnt1p, because transformation of rnt1Δ cells with a plasmid harboring RNT1 restores normal telomere length (Fig. 2A).

We next examined whether this increase in telomere length is associated with an increase in telomerase activity. Relative telomerase activity was assessed in extracts derived from wild type and rnt1Δ cells expressing a protein A-tagged version of
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**Figure 2.** Rnt1p is required for normal telomerase activity. A, Southern blot analysis of telomere length. Genomic DNA was extracted from wild type or rnt1Δ cells and digested with XhoI restriction enzyme to release the terminal restriction fragment (TRF). The DNA was separated on a 1% agarose gel, transferred to a nylon membrane, and visualized with a randomly labeled telomeric probe. Note that this experiment was repeated several times using different clones and strain backgrounds and the increase in the telomere length was reproducible for each clone and not due to clonal variation. B, in vitro assay of the telomerase activity. Telomerase was partially purified by immunoprecipitating proA-EST2 using IgG affinity chromatography. RNA-dependent extension of a telomeric primer was monitored using denaturing PAGE. The increase in the relative telomerase activity (RTA) was calculated using the activity of the wild type telomerase as a reference. The data were obtained from three independent experiments using three independent cell extracts with a S.D. ≥ 0.8.

Est2p. In this procedure, telomerase was partially purified using IgG-Sepharose beads and used for a direct extension of a telomeric primer (44). As shown in Fig. 2B, deletion of RNT1 resulted in an increase in relative telomerase activity as compared with the internal control (Fig. 2B). The observed activity is telomerase specific, because it is RNA dependent and was not observed in precipitates from untagged cells. The observed increase in the telomerase activity is Rnt1p-dependent but strain background-independent and was also observed after the inactivation of a temperature-sensitive allele of RNT1 (data not shown). Thus, the accumulation of the RNAs of telomerase core subunits upon the deletion of Rnt1p leads to an increase in both its in vitro activity and telomere length.

Deletion of Rnt1p affects many cellular pathways and slows cell metabolism (34). To ensure that the increased levels of RNA of telomerase subunits is not a secondary effect generated by long term changes in cell metabolism, we monitored the kinetics of telomerase induction upon inactivating a temperature-sensitive allele of Rnt1p (rnt1-ts) (22). If the RNAs coding for the telomerase subunits are targets of Rnt1p-mediated RNA metabolism, their steady-state levels should change with the same kinetics at that of known Rnt1p substrates (e.g. snoRNAs). Total RNA was extracted from rnt1-ts cells at different intervals after shift to the restrictive temperature (37 °C). The extracted RNA was analyzed using Northern blots or real-time PCR. As expected, Northern blots probed with a snR43-specific probe, a known substrate of Rnt1p, documented a processing defect as early as 2 h after shift to 37 °C (Fig. 3A). Real-time PCR analysis of the same RNA samples indicated that similar to snR43, the levels of the different mRNAs are presented relative to that of Act1. The indicated values represent an average of three independent experiments. The graph curves were obtained using Microsoft Excel. C, comparison between the expression levels of the telomerase-related RNAs in wild type cells, rnt1Δ cells, and cells expressing a catalytically impaired version of Rnt1p (rnt1-D247R). The RNA was analyzed by Northern blot gene-specific probes. Expression levels were quantified using Instant Imager and normalized to Act1 mRNA as a loading control. The indicated values represent an average of three independent experiments with a S.D. ≥ 0.4 or less.
suggest that Est1, Est2, Est3, and Tlc1 RNAs are targets of Rnt1p-controlled RNA metabolism.

Rnt1p is an endoribonuclease that could influence the RNA levels of telomerase subunits by directly cleaving the respective RNAs thereby accelerating their degradation. To test this possibility, we monitored the impact on the expression of the telomerase subunits of a point mutation in RNT1 (D247R), which leads to an impaired Rnt1p RNA-cleavage activity without affecting other functions (45, 46). Total RNA was extracted from wild type, rnt1Δ, or rnt1-D247R cells and analyzed using Northern blots. As shown in Fig. 3C, RNAs extracted from rnt1Δ and rnt1-D247R cells exhibit similar increases in the mRNAs of telomerase subunits when compared with wild type cells. We conclude that the RNA-cleavage activity of Rnt1p is required for the regulation of normal expression levels of telomerase subunits.

Most Rnt1p substrates include a short RNA stem capped with a terminal AGNN tetraloop (27–29). We exploited this feature to search for potential Rnt1p substrates within the RNAs coding for telomerase subunits. This in silico search was performed using a previously established strategy that scores potential substrates based on similarity to known cleavage signals and other overall structural features (47). One stem-loop structure with high similarity to known substrates was predicted within the coding sequence of Est1 mRNA near its 3′ end (Fig. 4A). A model RNA representing the predicted cleavage signal was synthesized in vitro and tested for cleavage using purified recombinant Rnt1p. As shown in Fig. 4B, the Est1 RNA structure is cleaved by Rnt1p in vitro at the predicted distance from the tetraloop and mutations that disrupt the formation of the stem-loop structure (Est1–4) abolished the cleavage. We also tested the capacity of Rnt1p to cleave the predicted structure in the context of mRNA isolated from cells. Total RNA was extracted from wild type cells or cells expressing a version of the stem-loop structure (Est1–4) carrying silent mutations that disrupt the Rnt1p cleavage signal (Fig. 4A). The RNA was incubated with purified recombinant enzyme and the cleavage was detected by either Northern blot or primer extension. As shown in Fig. 4C, Est1 mRNA is cleaved by Rnt1p, releasing a product of ~2000 nucleotides, consistent with the cleavage of the predicted structure, whereas the mRNA for Est1–4 was not cleaved. Primer extension analysis mapped the cleavage sites of Rnt1p at 14 and 16 bp from the stem-loop as predicted (data not shown). Examination of the EST1 sequence from other species revealed the presence of a potential Rnt1p cleavage site in all Saccharomyces species where the enzyme specificity is conserved (data not shown). To ensure that our in silico search did not miss any potential cleavage sites, we performed a similar in vitro cleavage assay on the RNAs coding for the other components of telomerase. This analysis did not reveal any Rnt1p-specific cleavage in vitro (data not shown). These results suggest that Est2, Est3, and Tlc1 RNAs do not contain canonical Rnt1p cleavage sites, but they do not exclude the possibility that these RNAs are cleaved by Rnt1p using an alternative mechanism in vivo. We conclude that the mRNA coding for Est1p is a direct substrate of Rnt1p.

Most known mRNA substrates of Rnt1p are cleaved by this enzyme in response to variation in growth conditions or cellular signals (22, 24). Therefore, we presumed that the cleavage of the Est1 RNA in vivo might also be triggered by a specific signal. The function of Est1p is cell cycle regulated and there is evidence that the protein is associated with active telomerase at the telomeres in late S phase (14). A small reduction of Est1 mRNA expression has also been observed in G1–arrested cells (48). We therefore hypothesized that Est1 mRNA stability could be regulated in a cell cycle- and Rnt1p-dependent manner. To test this hypothesis, we monitored the expression level of the Est1 mRNA during the cell cycle using synchronized cell cultures of wild type cells (EST1; bar1Δ) and cells expressing the version of Est1 mRNA that is not cleaved by Rnt1p (EST1–4; bar1Δ). EST1; bar1Δ and EST1–4; bar1Δ cells were synchronized in G1 phase of the cell cycle with α-factor and after release, cell cycle progression was monitored by FACS analysis (Fig. 5A). As documented by the FACS profiles, a proportion of the cells were delayed in re-entering the cell cycle.

**FIGURE 4.** Rnt1p directly cleaves Est1 mRNA using a conserved cleavage signal. A, schematic representation of the cleavage signal of Rnt1p at the 3′ end of Est1 mRNA. The RNA structure was predicted in silico using Mfold (59). The predicted cleavage sites of Rnt1p are indicated by arrows. P1 and P2 indicate the cleavage products observed in vitro. The boxed letters indicate the mutations that disrupt Rnt1p cleavage signals. The underlined letters indicate the position of the third nucleotide in the codon of Est1 open reading frame near the tetraloop recognized by Rnt1p. Bold italic nucleotides represent the 5′ and 3′ ends of the synthetic substrate used for in vitro cleavage assay in B. The nucleotide highlighted in gray was introduced to stabilize the synthetic substrate. B, in vitro cleavage of a model Est1 substrate. Model RNA substrate representing the predicted Rnt1p cleavage site within the coding sequence of the Est1 mRNA (EST1) or a version carrying mutations disrupting Rnt1p recognition site (EST1–4) were T7 transcribed and incubated in vitro with (+) or without (−) recombinant Rnt1p enzyme in the presence of 150 mM KCl and MgCl2 at 30 °C. The position of the substrate (S) and the products (P1 and P2) are indicated on the right. The position of the 10 bp ladder (M) is indicated on the left. C, cleavage of total RNA extracted from wild type cells (RNT1; EST1) or cells expressing a version of Est1 mRNA carrying the 4 silent mutations (indicated in A) that disrupt the tetraloop structure (RNT1; EST1–4). RNA was incubated with (+) or without (−) recombinant Rnt1p enzyme as described in B. The position of the full-length mRNA and the 5′ end cleavage product (P) are indicated on the right. The position of the predicted cleavage site was confirmed by primer extension (data not shown) and indicated in A.
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As expected, the mRNA of the cell cycle-regulated gene EST1 displayed similar expression patterns in both G1 and S phases of the cell cycle (Fig. 5A). The cell cycle-dependent induction of EST1 mRNA, which carries mutations disrupting Rnt1p cleavage site, was similar to that of the EST1 mRNA in S phase. However, at the end of the S phase, more EST1 mRNA than EST1 mRNA was detected. As observed in Fig. 5B, the highest detected EST1 mRNA value was in EST1; bar1Δ strains, whereas the lowest EST1 mRNA value was significantly higher in EST1–4; bar1Δ strains compared with the EST1; bar1Δ strain. As expected, no difference was observed for EST1 mRNA between the two strains. We conclude that the identified Rnt1p cleavage of the EST1 mRNA contributes to efficient cell cycle-dependent repression of EST1 mRNA.

DISCUSSION

In this study, we show that yeast RNase III negatively regulates the expression of yeast telomerase subunits and contributes to the cell cycle regulation of EST1 mRNA level. Decreased decay rates and increased steady-state levels of the RNAs lead to an increase in telomerase activity and to telomere elongation (Figs. 1 and 2). The catalytic endoribonucleolytic activity of Rnt1p was required for controlling the expression of the telomerase RNP complex, suggesting that Rnt1p targets one or more telomerase-related RNAs for direct cleavage (Fig. 3). Indeed, recombinant Rnt1p cleaved EST1 mRNA in vitro and mutations that disrupt Rnt1p cleavage impaired the cell cycle repression of EST1 mRNA without affecting the decay of other cell cycle-regulated RNAs (Figs. 4 and 5). These results reveal a new layer of regulation that controls telomere length by adjusting the expression of telomerase subunits and identify new targets for eukaryotic RNase III.

Efforts to understand how telomere length is regulated revealed a cell cycle controlled machinery that encompasses competing telomere elongation and shortening factors (50, 51). However, very little is known about how the expression of telomerase itself is regulated and what impact variations in its expression levels may have on the telomere length. Earlier studies indicated that the expression levels of individual components of the telomerase RNA complex are not interdependent because an overexpression of any single factor appeared not to alter the expression of the others (9, 52, 53). It was proposed that an increase in the telomerase activity requires increased expression of at least the majority of the components of the telomerase RNP complex (52). However, conditions that change these expression levels accordingly and factors that regulate them were not identified thus far. The present study identifies Rnt1p as such a factor. The increase in telomerase activity upon the deletion of RNT1 is not a generic response to general perturbation in RNA metabolism (Fig. 3). In addition, previous studies have shown that mutations inhibiting nonsense-mediated mRNA decay induces the expression of EST1, EST2, and EST3 RNAs, but, unlike deletion of RNT1, lead to short telomeres (11). The simultaneous induction of the different telomerase subunit RNAs in the absence of RNT1 indicates that either Rnt1p controls the expression of each RNA independently or it induces the expression of a single RNA that in turn induces the
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others. We prefer the first possibility because expressing the subunit RNAs individually from a heterologous and repressible promoter did not affect the expression of the other subunits.\(^5\) Consistently, a mutation that only disrupts Rnt1p cleavage of Est1 mRNA does not increase the expression of the other components of the telomerase holoenzyme (data not shown).

The results thus raise the question of how Rnt1p exerts this negative regulation on the expression levels of the mRNAs coding for telomerase subunits. One possibility is that Rnt1p simultaneously cleaves Est1, Est2, Est3, and Tlc1 RNAs in the nucleus, before they are exported to the cytoplasm (54). This is supported by the fact that the catalytic activity of Rnt1p is required for the normal expression of all four RNAs and that the response of these RNAs to the inactivation of Rnt1p follows the same kinetics as that of known substrates. Moreover, our data do show that Est1 mRNA can directly be cleaved by Rnt1p in vitro (Fig. 4). Our failure to detect such direct cleavage of Est2, Est3, and Tlc1 RNAs could be explained if those RNAs require additional cellular factors for cleavage. Previous studies have shown that chaperones may mediate the cleavage of non-canonical Rnt1p substrates (55). For example, processing of the snoRNA U18 is Rnt1p-dependent in vivo, but recombinant Rnt1p alone does not cleave pre-U18 snoRNA in vitro (55). Such an in vitro cleavage of pre-U18 requires the presence of Nop1p, a nucleolar protein that associates with U18. It is therefore possible that yet to be identified proteins mediate the cleavage of Est2, Est3, and Tlc1 by Rnt1p in a U18-like fashion. Another possibility is that Rnt1p cleaves another RNA that will affect telomerase mRNA levels. Because the catalytic activity of Rnt1p is required for normal telomerase mRNA levels, that possibility could be envisaged but no such example has been observed yet.

The discovery that Rnt1p directly cleaves Est1 mRNA adds to a growing list of Rnt1p substrates that include mRNAs coding for glucose and iron-related proteins (22, 24). To date, yeast is the only eukaryote in which direct cleavage of mRNAs by RNase III enzymes has been documented. In vertebrates, conditional mRNA degradation is normally carried out in the cytoplasm by the machinery of RNA interference (56, 57). The fact that Rnt1p is localized in the nucleus suggests that it cleaves its target mRNAs in the nucleoplasm, which raises questions about the function of this cleavage and its contribution to the overall regulation of gene expression (34). Nascent mRNAs are exported rapidly to the cytoplasm and at any given time, the bulk of cellular mRNAs are found in the cytoplasm (54). This explains why deletion of RNT1 or disruption of Rnt1p cleavage activity does not dramatically increase the half-life of its target RNAs. Similarly, a disruption of its cleavage site in the targeted RNA, like in the case of the Est1–4, does not lead to a major increase of the RNA steady-state level. This observation is consistent with those made in previous studies using other Rnt1p mRNA targets (22, 24). It is therefore unlikely that the function of Rnt1p cleavage is to fully silence the expression of EST1 or similarly regulated genes. Instead, Rnt1p-dependent cleavage may provide a fail-safe mechanism for programmed transcrip-

\(^5\) S. Larose, R. J. Wellinger, S. Abou Elela, unpublished observation.

It is known that the abundance of Est1p is cell cycle regulated (14). Consistent with recent results (48) and with previous genome-wide transcriptional profiling (49), we show here that this is accompanied by a cell cycle-dependent regulation of the mRNA coding for Est1p. A significant increase of Est1 mRNA was observed during the transition from G₁ to S phase of the cell cycle. This increase correlates with an increase of the protein level observed in S phase (14). After S phase and when cells enter G₂, the level of Est1 mRNA rapidly decreased (Fig. 5). It is noteworthy that the normally strictly nucleolar localization of Rnt1p is relaxed exactly at this point in the cell cycle such that the enzyme is found throughout the nucleus in G₂/M (34). This cell cycle regulated control of Rnt1p localization could thus be responsible for the cell cycle-dependent degradation of the Est1 mRNA. A comparable situation was previously reported for the endoribonuclease MRP: the exit of this enzyme from the nucleolus allows the cleavage of the cell cycle-regulated Clb2 mRNA (58). The G₁/S phase-dependent increase in Est1 mRNA therefore occurs when Rnt1p is sequestered in the nucleolus and consequently is unchanged, even if Rnt1p cleavage site is disrupted in the Est1–4 mRNA. This implies that transcription of EST1 itself is induced in a cell cycle-dependent manner and corroborates the inclusion of this gene in the list of G₁/S-induced genes that encompasses several DNA-replication genes (49). The G₂-dependent decrease of the Est1 mRNA level was not blocked by the deletion of RNT1, suggesting that normal decay of the mRNA combined with reduced transcription contribute to Est1 repression. Therefore, our results indicate that telomerase activity in yeast is regulated by a combination of transcriptional and post-transcriptional events.

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REFERENCES

1. Hug, N., and Lingner, J. (2006) Chromosoma (Berl.) 115, 413–425
2. LeBel, C., and Wellinger, R. J. (2005) J. Cell Sci. 118, 2785–2788
3. Lundblad, V., and Szostak, J. W. (1989) Cell 57, 633–643
4. Greider, C. W., and Blackburn, E. H. (1985) Cell 43, 405–413
5. Cohn, M., and Blackburn, E. H. (1995) Science 269, 396–400
6. Lingner, J., Cech, T. R., Hughes, T. R., and Lundblad, V. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11190–11195
7. Lendvay, T. S., Morris, D. K., Sah, J., Balasubramanian, B., and Lundblad, V. (1996) Genetics 144, 1399–1412
8. Singer, M. S., and Gottschling, D. E. (1994) Science 266, 404–409
9. Peterson, S. E., Stellwagen, A. E., Diede, S. J., Singer, M. S., Haimberger, Z. W., Johnson, C. O., Tzoneva, M., and Gottschling, D. E. (2001) Nat. Genet. 27, 64–67
10. Virta-Pearlman, V., Morris, D. K., and Lundblad, V. (1996) Genes Dev. 10, 3094–3104
11. Dahlseid, J. N., Lew-Smith, J., Lelivelt, M. J., Enomoto, S., Ford, A., Desruisseaux, M., McClellan, M., Lue, N., Cubertson, M. R., and Berman, J. (2003) Eukaryotic Cell 2, 134–142
12. Diede, S. J., and Gottschling, D. E. (1999) Cell 99, 723–733
