mRNAs Encoding Telomerase Components and Regulators Are Controlled by UPF Genes in Saccharomyces cerevisiae

Jeffrey N. Dahlseid,1† Jodi Lew-Smith,2‡ Michael J. Lelivelt,3 Shinichiro Enomoto,2 Amanda Ford,3 Michelle Desruisseaux,2 Mark McClellan,2 Neal Lue,4 Michael R. Culbertson,5 and Judith Berman2,5*

Department of Chemistry, St. Olaf College, Northfield, Minnesota 55057; Department of Genetics, Cell Biology and Development2 and Department of Microbiology,3 University of Minnesota, Minneapolis, Minnesota 55455; Laboratories of Genetics and Molecular Biology, University of Wisconsin, Madison, Wisconsin 53706; and Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, New York 10021

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In Saccharomyces cerevisiae, average telomere length and telomeric silencing are reduced by loss of function of UPF genes required in the nonsense-mediated mRNA decay (NMD) pathway. Because NMD controls the mRNA levels of several hundred wild-type genes, we tested the hypothesis that NMD affects the expression of genes important for telomere functions. In upf mutants, high-density oligonucleotide microarrays and Northern blots revealed that the levels of mRNAs were increased for genes encoding the telomerase catalytic subunit (Est2p), in vivo regulators of telomerase (Est1p, Est3p, Stn1p, and Ten1p), and proteins that affect telomeric chromatin structure (Sas2p and Orc5p). We investigated whether overexpressing these genes could mimic the telomere length and telomeric silencing phenotypes seen previously in upf mutant strains. Increased dosage of STN1, especially in combination with increased dosage of TEN1, resulted in reduced telomere length that was indistinguishable from that in upf mutants. Increased levels of STN1 together with EST2 resulted in reduced telomeric silencing like that of upf mutants. The half-life of STN1 mRNA was not altered in upf mutant strains, suggesting that an NMD-controlled transcription factor regulates the levels of STN1 mRNA. Together, these results suggest that NMD maintains the balance of gene products that control telomere length and telomeric silencing primarily by maintaining appropriate levels of STN1, TEN1, and EST2 mRNA.

Telomeres, the ends of linear chromosomes, are important for chromosome integrity and are maintained by telomerase, a reverse transcriptase-like enzyme that includes an integral RNA template. The catalytic components of Saccharomyces cerevisiae telomerase (TLC1 RNA and Est2p), as well as gene products required for telomerase activity in vivo (e.g., Est1p, Est3p, Cdc13p, Cdc4p, Ku70/80, Mec1p, MRX, Rcp1p, Stn1p, Tel1p, and Ten1p), have been identified (reviewed in reference 12). However, mechanisms that regulate the expression and activity of telomerase components and modulators have not been fully explored.

The nonsense-mediated mRNA decay (NMD) pathway accelerates the degradation of mRNAs that prematurely terminate translation due to nonsense mutations, frameshifts, or translation of alternate open reading frames (ORFs) within the mRNA (21, 37). In S. cerevisiae, the products of UPF1, UPF2, and UPF3 are required for NMD and provide a surveillance function to lower the abundance of potentially deleterious protein fragments by degrading mRNAs that cannot be translated full length (42). However, the only known growth phenotype of upf mutants is deficient respiration (1). Interestingly, NMD also controls the expression of some wild-type genes. By using high-density oligonucleotide arrays (HDOAs), several hundred wild-type S. cerevisiae mRNAs with either increased or decreased steady-state levels in upf mutants were identified (23). NMD directly regulates the level of wild-type mRNAs for some genes, such as SPT10 and CPA1, through accelerated degradation triggered by translation of alternate ORFs within the mRNA (46, 50). Given the large number of genes controlled by NMD, including transcription factors such as Prp1p (29, 45) and Ino4p (23), many wild-type mRNAs are likely to change in abundance as an indirect consequence of changes in the abundance of transcriptional regulators (23).

Previously it has been found that mutations in UPF1, UPF2, or UPF3 reduced telomere length and silencing of a telomere-adjacent reporter gene (25). It was hypothesized that NMD regulates telomeres by altering the levels of specific wild-type mRNAs important for telomere functions. To identify genes important for the telomere-related phenotypes of upf mutants, we screened the HDOA data of Lelivelt et al. (23) for S. cerevisiae genes thought to be important for telomere functions. Here we report that mRNAs encoding the catalytic subunit of telomerase, regulators of telomerase activity, and proteins that affect telomeric silencing are all controlled by NMD. Furthermore, extra copies of EST2, STN1, and TEN1 were

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TABLE 1. Expression of selected genes with possible telomere-related functions in upf mutant strains relative to that in UPF strains

| ORF       | Gene name | CKI | AFC |
|-----------|-----------|-----|-----|
| YLR010C   | TEN1      | 1.00| 3.00|
| YMR127C   | SAS2      | 0.88| 3.20|
| YNL261W   | ORC5      | 0.67| 1.75|
| YDR082W   | STN1      | 0.59| 2.55|
| YBR195C   | MSU1/CAC3 | 0.56| 2.29|
| YLR318W   | EST2      | 0.56| 2.94|
| YLR233C   | EST1      | 0.53| 2.25|
| YCL011C   | RL6F      | 0.44| 1.30|
| YMR106C   | HDF2      | 0.25| 1.40|
| YML061C   | PIP1      | 0.22| 2.05|
| YLR453C   | RIF2      | 0.22| 1.90|
| YMR284W   | HDF1      | 0.19| 1.53|
| YPR018W   | CAC1/RLF2 | 0.13| 0.25|
| YPL001W   | HAT1      | 0.13| 0.98|
| YLR223C   | IFH1      | 0.13| 0.98|
| YNL250W   | RAD50     | 0.13| 7.03|
| YPL128C   | TBF1      | 0.13| 3.70|
| YGR099W   | TEL2      | 0.13| 1.02|
| YML102W   | CAC2      | 0.09| 1.06|
| YMR224C   | MEE11     | 0.09| 1.32|
| YPR162C   | ORC4      | 0.09| 1.46|
| YPL153C   | RAD53     | 0.09| 4.60|
| YKR101W   | SIR1      | 0.09| 1.42|
| YOR351C   | MEK1      | 0.07| 1.07|
| YOL051W   | GAL11     | 0.06| 1.13|
| YEL056W   | HAT2      | 0.06| 1.13|
| YOR025W   | HST3      | 0.06| 1.33|
| YDR191W   | HST4      | 0.06| 1.26|
| YNL330C   | RPD3      | 0.06| 1.03|
| YDR369C   | XRS2      | 0.06| 1.03|
| YNL102W   | CDC17     | 0.03| 1.15|
| YOR058C   | HIR2      | 0.03| 0.98|
| YGL058W   | RAD6      | 0.03| 1.06|
| YBL086C   | TEL1      | 0.03| 1.12|
| YOR229W   | WTM2      | 0.03| 0.87|
| YDL220C   | CDC13     | 0.00| 1.14|
| YOR217W   | CDC44     | 0.00| 0.99|
| YER088C   | DOT6      | 0.00| 1.08|

a CKI (combined knockout index) was defined by Lealveldt and Culbertson (23). For each mRNA, numerical weights were assigned to GeneChip difference calls for four trials for each of the four upf mutant strains compared to four trials for an isogenic UPF strain as follows: increased signal (+2), statistically marginal increase (+1), no change (0), statistically marginal decrease (−1), and decrease (−2). The numerical values of the difference calls were summed across all trials and then divided by the maximum potential score to yield the CKI score. Scores of ±0.5 indicate that the given mRNA was in abundance with relative consistency from trial to trial, whereas scores of ±0.5 that a given mRNA was decreased in abundance with similar relative consistency.

b AFC (average fold change) was measured independently of the CKI score. The AFC is the average increase or decrease in mRNA abundance from 16 trials with upf mutants compared with four trials with UPF strains. AFCs for some mRNAs with CKI scores between 0.5 and −0.5 are not reliable because the CKI score indicates poor consistency of calls across trials.

sufficient to mimic the telomeric silencing and telomere length phenotypes of upf mutants.

MATERIALS AND METHODS

Yeast strains, plasmids, and growth conditions. The yeast strain used for HDOA studies was LRS3/07 (MATa his3-11,15 leu2-3,112 trp1-289 ade2-100). The yeast strain used for the telomere length assays, telomerase assays, and plasmid protection experiments was performed on strain YJB209 (MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1, ura3-1) transformed with appropriate plasmids and strain YJB195 (MATa ura3-1 ade2-1 his3-11,15 leu2-3,112 can1-100 trp1-1) transformed with YEpLac81, p2μ-STM1a, and p2μ-EST2, respectively, as described below and in the figure legends. RNA half-life experiments were conducted using strain AAY333 (MATa ADE2 ura3 his3-11,15 trp1-1 leu2-3,112 ade2-1 ura3-1) transformed with either pRS315 or pRS315-UPF1. Plasmid p2μ-EST1 (pVL157; provided by V. Lundblad) contains a 2.58-kb BamH1/Sph1 fragment of EST1 inserted in the BamH1/Sph1 site of YEp24. p2μ-EST2 contains a 3.75-kb BamHI-Sac1 fragment containing the entire coding sequence of EST2 in YEpLac81 (15). p2μ-EST3 contains a 1.15-kb fragment containing the wild-type EST3 gene in YEpLac81. p2μ-STM1a contains full-length STM1 cloned as a Pst1/Sac1 fragment into the Sma1-Sac1 sites in pRS425 (7). p2μ-STM1b contains full-length STM1 cloned into YEpLac395 (provided by M. Charbonneau), p2μ-STM2 (pDR1058; provided by D. Rivier) contains full-length STM2 cloned into pRS425. p2μ-ORC5 (pAD002; provided by A. Dillon and J. Rine) contains ORC5 cloned as a Xho1-Sac1 fragment into pFAT1. p2μ-ORC5 (provided by M. Charbonneau) contains the controlled gene in the Xho1-Sac1 fragment into pFAT1. p2μ-ORC5 (provided by M. Charbonneau) contains the stimulated gene in the CHS1::HIS3 module.

Strains expressing green fluorescent protein (GFP) reporter fusion mRNAs
were constructed by PCR-mediated homologous recombination (49). Each ORF was replaced from the start codon to the stop codon with the complete GFP ORF. P<sub>FST</sub>-GFP, P<sub>FST</sub>-GFP, and P<sub>FST</sub>-GFP were made by using pVL368, pVL296, and pVL298 (all provided by V. Lundblad), respectively, as templates. P<sub>FST</sub>-GFP was made by using pSE1593, which contains the STN1 gene in YePlac181.

**Telomere length and yeast telomerase assays.** Average telomere length was determined by Southern blot analysis of PsI-digested genomic DNA as described previously (25). To assess telomerase activity, yeast whole-cell extracts and DEAE fractions were prepared as previously described (8, 32). DEAE fractions were tested for telomerase activity by using two different primers in standard primer extension assays, and the results were quantified by PhosphorImager analysis (32).

**RNA methods.** For all life and steady-state experiments, total RNA was extracted as described by Lees et al. (21). To measure mRNA half-lives, strain AAY333 (provided by A. Akin, University of Nebraska, Lincoln), which carries the rpb1<sup>T</sup> temperature-sensitive allele encoding RNA polymerase II (4), was used. Transcription was terminated by shifting cells from 25 to 37°C. Cells were collected at intervals following temperature shift. After extraction, RNA was denatured by using glyoxal and dimethyl sulfoxide, separated on 1% agarose gels, transferred to GeneScreen Plus (Dupont, NEN Research Products, Boston, Mass.) (4). RNA was detected by hybridization to radiolabeled DNA probes or riboprobes, which were prepared by in vitro transcription of template DNA in the presence of [α-<sup>32</sup>P]UTP (800 or 3,000 Ci/mmol; Amersham Life Science, Arlington Heights, Ill.) by using the Riboprobe System (Promega Corp., Madison, Wis.). DNA templates for riboprobe synthesis were prepared by PCR amplification of genomic DNA by using standard conditions. The ACT1 riboprobe is 224 nt in length and contains sequences complementary to nt 30 to 528 of the ORF. The CDC13 riboprobe is 270 nt in length and contains sequences complementary to nt 2 to 271 of the ORF. The CPA1 riboprobe is 697 nt in length and contains sequences complementary to nt 706 to 139 of the ORF. The EST2 riboprobe is 645 nt in length and contains sequences complementary to nt 1011 to 1655 of the ORF. The EST3 riboprobe is 597 nt in length and contains sequences complementary to nt 29 through 568 relative to the first nucleotide of the ORF. The GFP riboprobe is 363 nt in length and contains sequences complementary to nt 222 to 584 of the ORF. The ORC5 riboprobe is 430 nt in length and contains sequences complementary to nt 506 to 935 of the ORF. The PGK1 riboprobe is 95 nt in length and contains sequences complementary to nt 1359 to 1251 of the ORF. The SAS2 riboprobe is 308 nt in length and contains sequences complementary to nt 353 to 660 of the ORF. The STN1 riboprobe is 377 nt in length and contains sequences complementary to nt 551 to 927 of the ORF. The TEN1 riboprobe is 366 nt in length and contains sequences complementary to nt 2 to 344 of the ORF. By using template DNA fragments obtained from restriction digestion or PCR, CHY2 or TRP1 probes were prepared and hybridized as described previously (2).

For analysis of TEL1 RNA levels in telomerase fractions, the TEL1 gene (nt 1 to 1301) was amplified by PCR and cloned between the BamHI end sites of pBluescript II KS<sup>+</sup>. The resulting plasmid was linearized by digestion with HinfI, and antisense RNA encompassing residues 1097 to 1301 of the TEL1 gene was generated by T3 RNA polymerase in the presence of 12 μM [α-<sup>32</sup>P] GTP (31). Total RNAs from DEAE fractions were isolated and combined with the probe (100,000 cpm), precipitated with ethanol, hybridized, digested with RNase T<sub>1</sub>, RNase A, and proteinase K, and analyzed by gel electrophoresis (33).

**RESULTS AND DISCUSSION**

**Steady-state levels of EST1, EST2, EST3, STN1, TEN1, SAS2, and ORC5 mRNA are elevated in upf mutants.** We hypothesized that NMD regulates the steady-state mRNA levels of specific wild-type genes that are important for telomere function (25). To identify wild-type mRNAs that accumulate in upf mutant strains and are responsible for the associated telomere-related phenotypes, we focused on HDOA for a subset of ~80 ORFs that encode proteins with known or suspected telomere function (Table 1) (23). Those with a combined knockout index (CKI) score of >0.5 or <−0.5 were selected for analysis. The CKI score is an indicator of how consistently the level of a specific mRNA is elevated or decreased in upf mutant strains relative to that in an isogenic UPF parental strain (23). By this criterion, the mRNA levels of the majority of the selected set of telomere-related mRNAs were not significantly affected in upf mutants. However, 7 of the ~80 mRNAs (encoded by EST1, EST2, STN1, TEN1, SAS2, ORC5, and MSI1/CA43) had CKI scores of >0.5, with the average change in mRNA levels in upf mutants being an increase of 1.75- to 3.3-fold relative to the levels in UPF strains (Table 1). None of the mRNAs had a score of <−0.5. To confirm the results of the HDOA studies and to extend them to relevant RNAs not present on the HDOA, we compared steady-state levels of mRNAs in total RNA prepared from wild-type and mutant strains on Northern blots. As predicted from the HDOA results and to extend them to relevant RNAs not included on the HDOA because it does not have an obvious ORF (47), high levels of TEL1 RNA, like upf mutations, cause telomere shortening and reduced telomeric silencing (47). Northern analysis of total RNA indicated that TEL1 RNA levels were not affected by upf mutations (Table 2), which is consistent with the idea that the NMD pathway acts on mRNAs during translation (2, 3) and not on untranslated RNAs like the product of TEL1. Thus, despite the similarity in the phenotypes of upf mutants and strains expressing high

### Table 2. Average increases (n-fold) in mRNA levels measured by HDOA and Northern blot analysis

| Gene name | HDOA data<sup>a</sup> | Northern blotting<sup>b</sup> (upf1 upf2 upf3/UPF1 UPF2 UPF3)<sup>c</sup> | Northern blotting<sup>d</sup> (upf1/UPF1)<sup>e</sup> | GFP reporter Northern blotting<sup>f</sup> (upf3/UPF3) <sup>g</sup> |
|-----------|-----------------|---------------------------------|-----------------|---------------------------------|
| EST1      | 2.1 ± 0.4       | 2.8 ± 0.4                       | 3.6              | 2.1 ± 0.61                      |
| EST2      | 2.3 ± 1.1       | 4.3 ± 0.4                       | 7.7              | 1.8 ± 0.01                      |
| EST3      | NT              | 3.1 ± 0.3                       | 4.7              | 2.0 ± 0.01                      |
| ORC5      | 1.7 ± 0.3       | 2.0 ± 0.3                       | 2.6              | ND                              |
| SAS2      | 3.2 ± 0.9       | 2.9 ± 0.4                       | 2.8              | ND                              |
| STN1      | 2.6 ± 0.5       | 5.2 ± 0.5                       | 6.1              | 3.1 ± 0.1                       |
| TEN1      | 3.3 ± 0.7       | 4.8 ± 0.7<sup>h</sup>           | ND<sup>i</sup>   | ND                              |
| CDC13     | 1.1 ± 0.7       | 1.1 ± 0.2                       | 1.1              | ND                              |
| MSI1      | 2.1 ± 1.0       | 1.3 ± 0.3                       | 1.4              | ND                              |
| TEL1<sup>l</sup> | NT          | 0.8 ± 0.3<sup>l</sup>           | 0.9              | ND                              |

<sup>a</sup> Values represent average fold change and sample standard deviation (Table 1).<sup>b</sup> mRNA levels were determined by using quantitative RNA blots like those shown in Fig. 1 for isogenic upf mutant strain LRSy507 (pRS316) compared with wild-type strain LRSy307 (pML1). Values represent average of data ± standard deviation from the four upf mutant strains relative to the wild type (n = 4). In all cases, mRNA levels were normalized to actin mRNA levels.<sup>c</sup> mRNA levels were determined by using quantitative RNA blots of strains ML51 (upf1 mutant) and ML34 (UPF1<sup>l</sup> ).<sup>d</sup> GFP reporter mRNA levels were determined by quantitative RNA blots of strains YBJ3758 and YBJ4468.<sup>e</sup> TEN1 and TEL1 mRNA levels were determined in strain YBJ1471 (upf2 mutant) and were normalized to levels of PGK1 mRNA.<sup>f</sup> ND, not done.
levels of TLC1 RNA, NMD does not affect telomere function by altering the steady-state levels of TLC1 RNA.

The EST3 ORF includes a programmed +1 frameshift (34) but was not included on the HDOA. Because EST3 includes a frameshift within the 5' 50% of the mRNA that bypasses a premature stop codon, it was a good candidate for an mRNA that is degraded by the NMD pathway (21, 22). The result of Northern blot analysis was consistent with this prediction: EST3 mRNA levels in upf mutants increased approximately three- to fivefold relative to those in the isogenic UPF strains (Table 2).

STN1 and TEN1 are essential genes and have a role in chromosome capping and the prevention of deleterious degradation of chromosome ends (18, 19). STN1 encodes a high-copy suppressor of cdc13-1 (19), and TEN1 encodes a gene product that interacts physically with both Cdc13p and Stn1p to enhance the ability of Stn1p to negatively regulate telomerase activity at telomeres (18). Northern blot analysis confirmed that the mRNA levels of STN1 and TEN1 were elevated in upf mutant strains (Table 2). Thus, the mRNA levels of several telomerase subunits and regulators (Est1p, Est2p, Est3p, Stn1p, and Ten1p) are influenced by the NMD pathway, while the RNAs for others involved in the same telomere-related processes (TLC1 RNA and Cdc13p) are not.

In addition to ORFs with known effects on telomerase function, data from the HDOA analysis revealed three genes (SAS2, ORC5, and MSI1/CAC3) with known effects on telomeric silencing and chromatin structure that had elevated mRNA levels in upf mutant strains. The results from Northern blot experiments were consistent with an increase in SAS2 and ORC5 mRNA levels of at least twofold (Table 2). Sas2p is a putative histone acetyltransferase that is a positive regulator of silencing at telomeres (44). ORC5 encodes a component of the origin recognition complex (ORC) (10, 28) and influences silencing at telomeres and the HM loci (14). MSI1/CAC3 mRNA, which encodes a subunit of chromatin assembly factor I, exhibited only a 1.3-fold increase on the Northern blots compared with the 2.1 ± 1.0-fold increase measured with HDOAs (Table 2). Therefore, we did not study MSI1/CAC3 further. Also consistent with the HDOA data, the levels of RAP1, SIR3, SIR4, histone H4 (HHF1 and HHF2), and CAC1/RLF2 mRNAs in upf mutant strains did not change (data not shown). Thus, the NMD pathway affects the accumulation of mRNAs for at least seven genes (EST1, EST2, EST3, STN1, TEN1, SAS2, and ORC5) that are important for telomerase activity, telomere length control, and/or telomeric silencing.

Elevated levels of Est2p and Stn1p together phenocopy the telomeric silencing defect of upf mutant strains. To test the hypothesis that the telomere-related phenotypes of upf mutants are caused by increases in the level(s) of one or more of the seven mRNAs (EST1, EST2, EST3, STN1, TEN1, SAS2, and ORC5), we investigated whether increasing the copy number of any one of the genes could mimic (phenocopy) the telomere-related phenotypes of upf mutants. Yeast 2 μm vectors were used to provide multiple copies of each of the individual genes with their native promoters. Northern analysis confirmed that the levels of the individual mRNAs increased twofold or more in strains carrying these 2μm plasmids (data not shown). Thus, the increased steady-state level of mRNA in these experiments generally equaled or exceeded the magnitude of the increase of specific mRNAs seen in upf mutants. Using strains that overexpress the gene products, we first examined whether increased copies of any individual gene could reduce the normal silencing of a telomere-adjacent gene. In wild-type strains, a URA3 gene inserted near the left end of chromosome VII is subject to epigenetic telomeric silencing such that a proportion of the cells are able to grow on 5-FOA (synthetic dextrose complete [SDC] plus 5-FOA), which is toxic to Ura+ strains. In upf mutant strains, the telomere-adjacent URA3 is no longer silent; thus, upf mutant cells do not grow on SDC plus 5-FOA (Fig. 1). An elevated level of EST2, EST3, STN1, SAS2, or ORC5 expression had no obvious effect on silencing of the telomeric URA3 gene in these otherwise wild-type strains (Fig. 1, rows a to h). Similar results were observed when extra copies of TEN1 or EST1 were provided and telomeric silencing was monitored by use of a strain in which ADE2 was inserted near the right end of chromosome V (data not shown). This is consistent with the observation that the overproduction of wild-type Est1p or Est2p does not affect telomeric silencing (13). Thus, an elevated level of any one of these mRNAs was not sufficient to account for the telomeric silencing phenotype of upf mutant strains.

We next tested whether overexpression of pairs of genes could confer a reduced silencing phenotype (similar to the
telomeric silencing phenotype of *upf* mutants). In each case, there was a greater-than-twofold average increase in the level of each RNA expressed (data not shown). Interestingly, only one combination of two plasmids, p2*μ*-STN1 with p2*μ*-EST2, resulted in reduced telomeric silencing (Fig. 1, rows i to s). In several independent transformants, telomeric silencing in strains containing both p2*μ*-STN1 and p2*μ*-EST2 (Fig. 1, row m) was similar to that seen in *upf* mutants, as indicated by an inability to grow in the presence of 5-FOA. Because the NMD pathway down-regulates *PPR1* (21, 38), which is a positive regulator of *URA3*, the slight difference in growth observed for these two strains is most likely due to increased levels of *PPR1* and *URA3* expression in the *upf* mutant strains relative to their levels in the *UPF* strain containing both p2*μ*-STN1 and p2*μ*-EST2. However, the effect of extra copies of p2*μ*-STN1 and p2*μ*-EST2 was seen when telomeric silencing was detected using ADE2 to mark the right end of chromosome V. Thus, the effect is not dependent on *URA3* or *PPR1*. We did not observe additional silencing when p2*μ*-TEN1 was expressed together with p2*μ*-EST2 or p2*μ*-STN1 by using the telomeric ADE2 marker on chromosome V (data not shown). Thus, extra copies of EST2 and STN1 together, which encode the catalytic subunit of telomerase and a regulator of telomerase function, respectively, were sufficient to recapitulate the telomeric silencing defect observed in *upf* mutant strains.

Our results support a connection between telomerase regulation and telomeric silencing and are consistent with the idea that titration of telomere-associated proteins influences telomeric silencing. *TLC1* was cloned as a high-copy disruptor of telomeric silencing (47). This occurs through a 48-nt stem-loop structure in *TLC1* RNA that most likely interacts with the Ku proteins (40), which are important for telomere organization within the nucleus as well as for telomeric silencing (20). Similarly, in *upf* mutant strains, high levels of Stn1p and Est2p may perturb telomeric silencing by altering the stoichiometry or function of telomere-associated proteins such as Cdc13p and/or Ku (17).

**Increased levels of Stn1p phenocopy the telomeric length control defect of *upf* mutant strains.** We next investigated whether additional copies of EST1, EST2, EST3, STN1, TEN1, SAS2, or ORC5 affected telomere length control to the degree seen in *upf* mutants. Telomere length was measured by digestion of genomic DNA with *Pst*I, which releases a terminal ~0.8-kb telomere fragment from the majority of the telomeres (those that contain a Y’ telomere-associated sequence [30]). Larger fragments that hybridize to the TG$_{1-3}$/C$_{1-3}$A probe correspond to non-Y’ telomeres and to subtelomeric fragments that include internal TG$_{1-3}$/C$_{1-3}$A repeats. The *upf* mutant strains carrying one or two control vectors exhibited an average telomere length that was ~57 bp shorter than that in an isogenic wild-type strain (Fig. 2) (Table 3). Transformation with p2*μ*-STN1 alone resulted in a consistent decrease in the lengths of the shortest terminal telomere fragments (~43 ± 19 bp shorter than those in the wild type [Fig. 2] [Table 3]), which were significantly different from the lengths of telomeres in the wild-type strain but not significantly different from the lengths of telomeres in *upf* mutant strains. In contrast, increasing the level of EST1, EST2, EST3, SAS2, ORC5, or TEN1 had no significant effect on telomere length in an otherwise wild-type strain (Fig. 2 and data not shown). Importantly, telomeres in strains that carried p2*μ*-STN1 together with either p2*μ*-TEN1 or p2*μ*-EST2 were 54 ± 26 bp and 52 ± 20 bp shorter, respectively, than wild-type telomeres (Fig. 2) (Table 3). When p2*μ*-STN1, p2*μ*-EST2, and p2*μ*-TEN1 were all present in the same strain, telomeres were 67 ± 28 bp shorter than in the wild-type and 10 ± 28 bp shorter than in the *upf* mutant strains. Since the levels of all three RNAs in this strain are higher than their levels in the *upf*2 mutant strain, this result suggests that increased levels of *STN1* are required for the short-telomere phenotype of *upf* mutant strains and that increased levels of *TEN1* and *EST2* mRNAs contribute to the phenotype. Consistent with this notion, both Stn2p and Ten1p are negative regulators of telomerase (17, 18).

Three NMD-sensitive genes, *STN1*, *TEN1*, and *EST2*, appear to account for the telomeric phenotypes of *upf* mutant strains. The effect of NMD on telomere length requires *STN1*, and the effect of NMD on telomeric silencing involves *STN1*...
and EST2. Thus, NMD confers the two phenotypes through increased levels of STN1 RNA in combination with other gene products. The involvement of Stn1p in both telomeric silencing and telomere length control is especially interesting in light of the proposed role for Stn1p as the primary effector of chromosome end protection (39). Through interactions with Cdc13p, Stn1p also has a role in coupling lagging-strand synthesis (of the telomeric C strand) to telomerase extension of the 3' end (telomeric G strand) of the chromosome (6). Thus, NMD-mediated control of the STN1 mRNA level appears to be critical for both the telomere length and telomeric silencing phenotypes of upf mutant strains.

Sequences necessary for NMD-mediated control of telomere-related mRNA abundance. The NMD pathway controls the levels of specific mRNAs either directly or indirectly. Direct effects, in which the decay rate of an mRNA is affected by NMD, result when an mRNA contains a built-in premature stop codon. In wild-type mRNAs that are normally subject to NMD, this may occur when a translatable upstream ORF is present in the 5' leader (46) or when leaky scanning leads to translation initiation at an out-of-frame AUG codon which affects on mRNA accumulation can result when the mRNA is transcriptionally regulated by the product of another mRNA that is affected by the NMD pathway (9, 23). Thus, mRNAs involved in telomere function could be either direct targets whose mRNA decay rates depend on NMD or indirect targets whose transcription rates depend on NMD.

We determined that NMD affects the expression of seven genes important for telomere functions and that a subset of these genes (EST2, STN1, and TEN1) can phenocopy a upf mutant strain. Because STN1 contributed significantly to both of the telomere-related phenotypes of upf mutant strains, experiments were done to address the effect of NMD on the STN1 mRNA levels. To determine whether STN1 mRNA is specifically targeted for degradation by NMD, we compared the half-lives of STN1 mRNA in wild-type and upf1 mutant strains. The half-lives of STN1 mRNA were not significantly different when measured in UPF1 and upf1 strains (Fig. 3A), whereas the control CPA1 mRNA (50) exhibited an approximately fourfold difference (Fig. 3C), indicating that STN1 mRNA does not appear to be a direct substrate of NMD. If STN1 mRNA is indirectly regulated by one or more NMD-sensitive transcription factors, then the STN1 promoter sequence is expected to confer NMD control to a reporter gene inserted in place of the STN1 coding sequence. To determine whether the STN1 promoter is sufficient for NMD control of the mRNA, we constructed a plasmid that fused 300 nt of DNA upstream to and including the STN1 start codon to the ORF for GFP (P_{STN1}-GFP) (27). Epifluorescence microscopy indicated that wild-type cells carrying P_{STN1}-GFP appeared slightly green due to expression of GFP from the STN1 sequence (data not shown). These cells were crossed to a upf3: HIS3 strain and sporulated. Epifluorescence analysis of ascis containing four spores revealed an apparent segregation of two bright green and two dim green spores (data not shown).

Northern blot analysis of RNA levels in sister spores from this cross (using a GFP riboprobe) indicated that levels of reporter

| Relevant genotype | Avg lengthb | Difference (bp) from: |
|------------------|-------------|----------------------|
|                  | WT          | upf2 mutant          |
|                  | 703 ± 21 (14) | 57                   |
| upf2             | 646 ± 9 (6)  | −57                  |
| STN1             | 660 ± 19 (6) | −43                  |
| STN1 TEN1        | 723 ± 23 (6) | −20                  |
| STN1 EST2        | 649 ± 26 (12) | −54                   |
| STN1 EST2 TEN1   | 651 ± 20 (6) | −52                  |
|                  | 636 ± 28 (6) | −67                  |

a Genotypes as described in the legend to Fig. 2. Genes shown in capital letters were provided on 2μm plasmids. b Average length in base pairs of PstI fragment ± standard deviation. The numbers in parentheses indicate the numbers of independent experiments done. c Not significantly different from the upf2 mutant according to the rank sum test (47). d Not significantly different from wild type according to the rank sum test (47).

![FIG. 3. STN1 and EST3 mRNA half-lives in wild-type and upf1 mutant strains](image-url)
mRNA were 3.1-fold higher in upf3 mutant strains than in UPF3 sister spores (Table 2). Analysis of isogenic upf3::HIS3 and UPF3 progeny by fluorimetry confirmed that upf3::HIS3 spores emitted significantly more GFP fluorescence than the UPF3 spores (data not shown). These results indicate that the STN1 promoter sequences are sufficient to confer NMD-dependent control on STN1 mRNA levels. Taken together, our results suggest that the promoter of STN1 is subject to control by NMD through an indirect mechanism involving the modulation of transcription levels. We propose that NMD controls the stability of an mRNA corresponding to an upstream regulator of STN1 expression.

Using GFP-reporter fusions, we compared EST1 and EST2 expression in wild-type and upf mutant strains. We constructed and analyzed the expression of a fusion construct containing EST1 sequence 5′ to the start codon fused to the GFP ORF. GFP fluorescence levels were too low for quantitation by epifluorescence microscopy, fluorimetry, or flow cytometry. Northern analysis revealed a twofold increase in the levels of reporter mRNA in upf3 spores relative to the levels in UPF3 sister spores (Table 2), suggesting that, like STN1, promoter sequences 5′ to the EST1 ORF contribute to the increased level of EST1 mRNA in upf mutant strains. Similar results were obtained when the EST2 ORF was replaced with the GFP ORF: the reporter mRNA levels were 1.8-fold higher in upf3 mutants than in UPF3 strains (Table 2). This indicates that sequences 5′ of the EST1 and EST2 ORFs are sufficient to account for all of the EST1 and most of the EST2 mRNA accumulation, respectively, in upf mutant strains relative to the levels in wild-type strains. However, we could not discern whether EST1 and EST2 mRNAs are regulated directly or indirectly, because the mRNA half-lives could not be measured.

The EST3 ORF was also replaced with that of GFP, and the accumulation of reporter mRNA levels was twofold greater in upf3 mutants than in UPF3 strains (Table 2), indicating that the increased level of EST3 mRNA in upf3 mutant strains was not due to sequences within the EST3 ORF. This was surprising because the EST3 ORF has an internal stop codon and a +1 programmed frameshift (34), suggesting that it might be degraded by NMD. Furthermore, the EST3 mRNA decay rates in upf1 mutant and UPF1 strains were similar (Fig. 3B), indicating that EST3 is not regulated at the level of mRNA decay. This implies that the internal stop codon in the ORF is not responsible for the effects of NMD on this mRNA. This in-frame stop codon could fail to trigger NMD for several reasons, including its position within the ORF relative to downstream sequences required for NMD or an interplay between the internal stop codon and the programmed frameshift site that allows translation to bypass the stop codon frequently. In either case, EST3 appears to be affected by NMD because of indirect effects on EST3 transcription initiation.

**Role of NMD in controlling the level of telomerase activity.** To determine whether telomere length in upf mutant strains was due to altered telomerase activity, we compared the ability of partially purified telomerase extracts prepared from wild-type and upf2 mutant strains to extend telomere sequence primers. Extracts from the two strains exhibited nearly identical activity in the primer extension assays with two different oligonucleotides as substrates (Fig. 4a). Consistent with the results of our Northern blot analysis of total RNA (Table 2), the amounts of TLC1 RNA in partially purified telomerase extracts were comparable in the two strains (Fig. 4B). Thus, loss of NMD (and the resulting increased levels of EST2 mRNA) does not affect the amount of extractable telomerase activity or the amount of TLC1 RNA in the telomerase fraction. In S. cerevisiae, in vitro telomerase activity requires Est2p and TLC1 but does not require Est1p or Est3p (26). Since neither in vitro telomerase activity nor the amount of TLC1 RNA is altered in upf mutant strains, it is possible that extractable telomerase activity in upf mutant cells may be limited by the levels of TLC1 RNA.

In vivo, EST1, EST3, STN1, and TEN1 contribute to telomerase-dependent telomere length control, presumably by regulating the access of the chromosomal terminus to telomerase (6, 17, 18, 39; reviewed in reference 12). Despite the fact that the NMD pathway controls levels of EST2 mRNA, which encodes the catalytic subunit of telomerase, telomere length and levels of extractable telomerase activity are not affected by

![FIG. 4. In vitro telomerase activity and TLC1 RNA levels are not dependent on UPF2.](image)

(a) TEL15

|        | wild-type | upf2 |
|--------|-----------|------|
| Recovery |           |      |

(b) Fraction

|        | wild-type | upf2 |
|--------|-----------|------|
| TLC1 RNA |           |      |


increased levels of EST2 mRNA (Fig. 2 and 3). Thus, the shorter telomeres in upf mutant strains are not due to increased levels of EST2 or to increased levels of telomerase activity. Rather, we propose that upf mutant strains have short telomeres because Stn1p (together with Ten1p) limits the accessibility of the telomeres to telomerase. Our results suggest that NMD affects telomeric silencing by increasing the levels of Stn1p and Est2p, which may titrate other factors that interact with the chromosome end complex and/or with telomerase itself. One candidate for the titrated factor is Cdc13p, which interacts with both telomerase and Stn1p (17, 39). Another candidate is the Ku70/Ku80 complex, which is also required for telomere length control and telomeric silencing (5, 20, 35, 41).

In summary, the level of expression of several telomerase components and regulators, including EST1, EST2, EST3, STN1, and TEN1, but not CDC13/EST4, depend on NMD. Increasing the levels of EST2, STN1, and TEN1, which encode the catalytic subunit of telomerase and two negative regulators of telomerase recruitment to the telomere, is sufficient to account for the telomeric silencing and telomere shortening phenotypes of upf mutants. While levels of mRNAs that regulate telomerase are altered in upf mutants, the levels of TLC1 RNA and of in vitro telomerase activity are not changed. This implies that the telomere length phenotype of upf mutants is due to changes in the access of telomerase to the telomere rather than to changes in the amount of telomerase activity. For EST1, EST2, EST3, and STN1, the sequences upstream of the ORF are sufficient to confer an NMD-mediated effect upon reporter mRNA levels. The effect of NMD on STN1 and EST3 mRNA levels most likely occurs via transcription initiation, since mRNAs were not stabilized in upf mutant strains. For EST3, this implies that the +1 programmed frameshift in the Est3p coding sequence (34) does not trigger UPF-mediated mRNA decay. We propose that NMD controls the mRNA stability for one or more upstream regulators of STN1 and possibly EST3. Thus, the NMD pathway affects telomere length and telomeric silencing by regulating the levels of EST2, STN1, and TEN1 mRNAs, primarily through an indirect effect on transcription levels.

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