Normal Telomere Length Maintenance in *Saccharomyces cerevisiae* Requires Nuclear Import of the Ever Shorter Telomeres 1 (Est1) Protein via the Importin Alpha Pathway

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The Est1 (ever shorter telomeres 1) protein is an essential component of yeast telomerase, a ribonucleoprotein complex that renews the repetitive sequences at chromosome ends (telomeres) that would otherwise be lost during DNA replication. Previous work has shown that the telomerase RNA component (*TLC1*) transits through the cytoplasm during telomerase biogenesis, but mechanisms of protein import have not been addressed. Here we identify three nuclear localization sequences (NLSs) in Est1p. Mutation of the most N-terminal NLS in the context of full-length Est1p reduces Est1p nuclear localization and causes telomere shortening—phenotypes that are rescued by fusion with the NLS from the simian virus 40 (SV40) large-T antigen. In contrast to that of the *TLC1* RNA, Est1p nuclear import is facilitated by Srp1p, the yeast homolog of importin α. The reduction in telomere length observed at the semipermissive temperature in a *srp1* mutant strain is rescued by increased Est1p expression, consistent with a defect in Est1p nuclear import. These studies suggest that at least two nuclear import pathways are required to achieve normal telomere length homeostasis in yeast.

Telomeres, the heterochromatic, G/T-rich regions of DNA located at the ends of linear chromosomes, are dynamic structures that undergo multiple rounds of attrition and elongation over the lifetimes of many eukaryotic cells. Because telomeres provide an essential capping function that protects DNA ends and aids in the maintenance of genomic stability, most eukaryotes use the enzyme telomerase to elongate telomeres (1).

Telomerase is a ribonucleoprotein complex in which the RNA subunit interacts with a specialized reverse transcriptase to synthesize telomeric DNA. In the yeast *Saccharomyces cerevisiae*, telomerase minimally consists of the *TLC1* RNA, which contains the template for nucleotide addition, and three ever shorter telomere (EST) proteins (2–5). Est2p is the reverse transcriptase that, together with *TLC1* RNA, is necessary and sufficient for enzyme activity in vitro (6, 7). Est1p and Est3p are essential regulatory components that stimulate the in vitro activity of telomerase and have been implicated in the recruitment and/or activation of telomerase at the telomere (5, 6, 8, 9).

Interactions between the subunits of telomerase and between telomerase and the telomere are complex. Est1p interacts with the single-stranded telomeric DNA binding protein, Cdc13p (10, 11). Ectopic expression of a Cdc13-Est2 fusion protein bypasses the requirement for *EST1*, suggesting that Est1p recruits telomerase to the telomere through the interaction with Cdc13p (12). *TLC1* RNA possesses distinct binding sites for Est1p and Est2p, suggesting that the interaction between Est1p and Est2p is mediated by the telomerase RNA in vivo (13–16). However, an RNA-independent interaction between Est1p and Est2p has been observed (8).

In live cells, persistent foci of *TLC1* RNA are detected at telomeres during S phase—a phenotype greatly reduced in cells harboring the *cdc13-2* mutation, in which telomere synthesis is perturbed (17). During G1 phase, Est2p is detected at telomeres by chromatin immunoprecipitation, in a manner that depends on the interaction of *TLC1* RNA with the DNA-end-binding yKu70/80p heterodimer (18–21). However, imaging of *TLC1* dynamics during G1 phase in live cells suggests that the interactions of *TLC1* with the telomere are transient and qualitatively different from those observed during S phase (17).

In contrast to Est2p and Est3p levels, Est1p protein levels are low in G2 phase, due to proteasome-mediated degradation (22, 23). Low levels of Est3p are detected at telomeres during G1 phase (24), presumably through the interaction of Est3p with Est2p (9), but the association of Est3p with telomeres increases in S phase, concurrent with rising Est1p expression and with the ability of telomerase to elongate telomeres (18, 19, 24, 25). Est1p is necessary and sufficient to stimulate the recruitment of Est3p to telomerase (22), consistent with the hypothesis that assembly of Est1p with telomerase allows optimal recruitment of Est3p to the complex.

Though much attention has focused on the dynamic associations of telomerase components with the telomere, less is known about where and when the components of telomerase assemble. By fluorescence in situ hybridization, it has been shown that endogenous *TLC1* RNA shuttles between the nucleus and the cytoplasm, with nuclear import depending on the β importins Mtr10p and Kap122p (26, 27). Furthermore, deletion of any one of the EST proteins or yKu70 perturbs *TLC1* RNA nuclear localization and/or retention (27). Despite what is known about *TLC1* RNA nucleocytoplasmic shuttling, direct studies of the subcellular lo-
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calization of telomerase protein components have been hampered by low protein abundance (24, 28, 29). The ability of other telomerase components to associate with telomeric DNA during G1 phase (when Est1p levels are low) suggests that Est1p may localize independently to telomeres. Indeed, overexpressed Est1p localizes to the nucleus even when present in great excess compared to other telomerase components (30, 31). These data support the idea that Est1p possesses a mechanism for nuclear import that is independent of its interactions with other components of telomerase and suggest that the regulation of Est1p nuclear import may contribute to telomerase biogenesis and function.

MATERIALS AND METHODS

Yeast strains. Standard protocols for manipulation of yeast were carried out as described previously (32). Strains and corresponding references are listed in Table S1 in the supplemental material; plasmids and corresponding references are listed in Table S2 in the supplemental material. The hygromycin resistance gene (HPHMX4) was PCR amplified from pBS4 by use of primers containing sequences found immediately upstream and downstream of the BAR1 open reading frame (ORF) (33), and the resulting product was transformed into yeast strain K1534 to generate YKF450. EcoRV linearization of Ylplac204/TcKdsRed-HDEL allowed for one-step integration of the construct into the TRPI locus of YKF450 to create YKF900. PCR amplification of the kanamycin resistance gene from pFA6a-KANMX6 by use of primers containing sequences found immediately upstream and downstream of the EST1 ORF generated a fragment that was transformed into YKF450 to produce YKF901. YKF902 was constructed in a similar manner, using sequences flanking KAP123. YKF903 was generated by PCR amplification of the kap122::KANMX4 locus from BY4741 kap122::KANMX4, followed by transformation of the PCR product into the mtr10-7 strain. Sequences of PCR primers used in this study are available upon request.

Plasmids. To generate pCH100, pSP809 (originally designed to insert ORFs at the C terminus of green fluorescent protein [GFP]) was altered to allow fusion at the N terminus of GFP. Briefly, the multiple-cloning site (MCS) at the C terminus of GFP was replaced with a stop codon. A DNA fragment containing the MCS, the GALI promoter, and the first 171 bp of the GFP ORF was generated by overlap extension PCR (34) and cloned into the AgeI/Msal sites of the redesigned pSP809 vector. GFP was replaced by enhanced GFP (S65T variant; EGFP) through PCR amplification from pCAT0604 and insertion into the HindIII/NotI sites of the redesi- gned pSP809 plasmid. To generate pCH100 (2GFP), EGFP was PCR amplified from pCH100 and inserted into the HindIII site of pCH100.

The EST1 open reading frame was PCR amplified from pRS416-EST1 and inserted into the SphI/NotI sites of pCH100 to generate pCH101. To fuse different regions of EST1 with 2GFP, pRS416-EST1 was used as the template to amplify regions of EST1 for cloning into the SphI/SphI sites of pCH100. EST1 mutants were created by site-directed mutagenesis within the N-terminal 600 bp or the central 900 bp of EST1 and cloned as BamHI/ PflMI or BspEI fragments, respectively, into pRS416-EST1. The resultant mutant vectors were used as templates to amplify specific regions of EST1 for cloning in frame into the BamHI/PflMI or BspEI sites of pCH100 or the SphI/SphI sites of pCH200.

The simian virus 40 large-T-antigen nuclear localization sequence (TAg-NLS) (35) (including residues GSPKKKKKEVAEEFGS; positively charged amino acids contributing to nuclear localization are underlined) was cloned into pRS416-EST1, pCH100, and pCH200 by annealing two oligonucleotides and inserting the resulting fragment into the BamHI site of each vector. The Nab2-NLS (36), containing residues 198 to 252 based on full-length Nab2p, was amplified from pAC719, and the 175-bp fragment was cloned into the BamHI/SphI sites of pCH101 to generate pCH112. To generate pCH015, a Spel/NotI fragment from pCH101 was inserted into the multiple-cloning site of pRS416. Next, a SacI fragment containing the EST1 terminator from pKF600 was inserted at the 3’ end of the EST1 ORF, and a PvuII/PflMI fragment from pRS416-EST1, containing the EST1 promoter as well as the first 717 bp of the EST1 coding sequence, was inserted.

To generate pCH016, a PvuII fragment from pRS416-EST1 was cloned into pRS413. After PCR amplification of SRP1 from genomic DNA iso- lated from strain YKF450, the 2,434-bp PCR product—containing 506 bp and 302 bp of SRP1 promoter and terminator sequences, respectively—was cloned into the Xhol/BamHI sites of pRS413 to generate pCH017. A BamHI/PflMI fragment from pCH112 was cloned into pCH016 to gener- ate pCH018.

Fluorescence microscopy. Direct fluorescence microscopy was used to examine the localization of GFP fusion proteins as well as dsRED- HDEL in YKF450-derived strains. Cells expressing GFP fusion proteins under the control of the GAL1 promoter were grown overnight to mid-log phase in synthetic complete medium lacking uracil and containing 2% raffinose. Galactose was added to a final concentration of 2%, and cells were incubated at 30°C for 1 h. Cells expressing GFP fusion proteins driven by a native promoter were grown similarly, without the addition of galactose. Hoechst 33342 was added to a final concentration of 1 μg/ml, and cells were incubated for 15 min at 30°C. Cells were washed once and resuspended in the appropriate expression medium (described above). Cells were imaged using a Zeiss Axios Observer inverted microscope (40× oil-immersion objective, 1.3 numerical aperture) with fluorescein isothiocyanate (FITC), Texas Red, and DAPI (4′,6-diamidino-2-phenylindole) filters (Semrock Brightline FITC-3540B-ZHE-ZERO, TXRED-4040B-ZHE-ZERO, and DAPI-1160A-ZHE-ZERO, respectively) and a Photometrics Cool Snap EZ charge-coupled device (CCD) camera. Images were acquired using Slidebook 4.2 software, making use of the “zoom +” feature, located under the Scope tab of the Focus Controls window, to obtain an additional ×2 magnification of the captured images. Images were collected and scaled using ImageJ software (37), and Adobe Photoshop CS5 software was used for image processing.

At least 100 GFP–expressing cells for each GFP fusion protein examined were quantified and binned as having a nuclear-only (N) phenotype, in which the fluorescence signal was localized exclusively in the nucleus; a cytoplasmic-only (C) phenotype, in which the fluorescence was localized primarily to the cytoplasm, with no evidence of nuclear enrichment; or an intermediate (I) phenotype, in which GFP fluorescence was both nuclear and cytoplasmic. N, I, and C are mutually exclusive designations. Cells were also categorized nonexclusively as having a vacuolar phenotype (V), in which GFP fluorescence was observed in the vacuole.

Strains containing temperature-sensitive alleles of importin mutants were grown to mid-log phase in appropriate selective media at the permissive temperature (18°C or 25°C), and galactose was added to the ap- propriate cultures to induce plasmid expression. A 3-ml aliquot was kept at the permissive temperature, while the remainder of the culture was shifted to the restrictive temperature (37°C). Cells were incubated for 5 h before a 30-min fixation by the addition of formaldehyde to a 3.7% final concentration and a 15-min DAPI-1160A-ZHE-ZERO, respectively) and a Hoechst staining step as described above. Cells were washed twice with 0.1 M potassium phosphate, pH 6.5, and resuspended in 1× phosphate-buffered saline prior to imaging. Because of the high level of cytoplasmic fluorescence associated with expression of Rnr4-GFPp from pMH1326, strains harboring this construct were incubated for only 2.5 h at the permissive temperature before fixation.

Telomere length analysis by Southern blotting. A YKF901 strain containing the complementing plasmid pRS416-EST1 was grown overnight in rich liquid medium and subsequently plated on solid medium containing 5-fluoroorotic acid (5-FOA; Gold Biotechnology) to select for loss of the complementing plasmid. A single YKF901 colony that grew on 5-FOA was inoculated into rich medium and transformed with variants of pRS416 or pCH101. ACY1563 was transformed with pRS413- or pRS423- derived constructs. Transformants were restreaked for ~100 generations on solid selective medium with 2% glucose, raffinose, or galactose as the carbon source, where appropriate. YKF901 strains were grown at 30°C, and ACY1563 strains were grown at 25°C or 35°C. Liquid cultures were

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grown to saturation in selective media at the appropriate temperature, and genomic DNA was isolated from each strain by glass bead lysis (38), digested with PstI, and separated in a 1.2% agarose gel. The DNA was blotted onto a Hybond N+ membrane (GE Healthcare), cross-linked to the membrane, and probed at 65°C using a yeast radiolabeled telomeric probe as previously described (39).

Southern blot images were quantified using ImageJ software. A line drawn down the middle of each lane was used to derive a plot of signal intensity at each lane position. Telomere restriction fragment (TRF) drawn down the middle of each lane was used to derive a plot of signal intensity at each lane position. Telomere restriction fragment (TRF) length was defined as the point of highest signal intensity within the predominant smear of Y′ telomeres and was converted to base pairs by comparison with a radiolabeled molecular weight ladder. In cases where a second smear of higher molecular weight was observed on the gel, the higher-molecular-weight smear was not included in the quantification. The derivation of this additional smear is unclear, but it could represent partial digestion. To account for slight differences in migration across the gel, where possible, samples were flanked by molecular weight marker lanes placed no more than 6 lanes apart. Marker bands of ≤4 kb were utilized for quantification. In cases where the flanking markers did not migrate identically, the lengths of intervening samples were corrected using the slope of a line connecting marker bands of the same molecular weight. Based on sequenced telomeres available in the Saccharomyces genome database (www.yeastgenome.org), the terminal PstI restriction site on Y′-element-containing telomeres lies an average of 540 bp from the TG₄₋₃ repeats of the yeast telomere. Therefore, telomere lengths were determined by subtracting 540 bp from each TRF length. Statistical analysis of the Southern blot data (analysis of variance [ANOVA] with Tukey’s post hoc test or Student’s t test) was performed using JMP software.

Fusion of the TAgNLS to wild-type EST1 slightly increased the telomere length compared to that in strains complemented with untagged EST1 alone. To account for this increase, the average difference in telomere length between the EST1- and TAgNLS-EST1-complemented strains was subtracted from the telomere lengths of strains harboring TAgNLS fusions with est1-mut1(FL), est1-mut2(FL), or est1-mut3(FL) prior to statistical analysis.

**Teleomere length analysis by ligation-mediated PCR.** After ACY1563 was transformed with pRS413- or pRS423-derived constructs, transformants were restreaked for ~150 generations at 25°C or 35°C on solid selective media. Liquid cultures were grown to saturation in selective media at the appropriate temperature. Genomic DNA was isolated from each strain by glass bead lysis (38) and prepared for ligation-mediated telomere PCR as described previously (40). In brief, after RNase treatment, genomic DNA was blunted with T4 DNA polymerase (New England Biolabs) and ligated to a double-stranded oligonucleotide, Y′-element-containing telomeres were amplified by PCR, using one primer that anneals to the subtelomeric DNAs of at least five yeast telomeres and a second primer that anneals to the ligated, double-stranded oligonucleotide. PCR products were stained with SYBR green (Life Technologies), resolved in a 10% Bis-Tris NuPAGE gel (Invitrogen) according to the manufacturer’s instructions and transferred to Hybond P membranes (GE Healthcare) by wet transfer in NuPAGE transfer buffer (Invitrogen). A 1:3,000 dilution of rabbit anti-GFP (Torrrey Pines Biolabs) was used as the primary antibody for GFP detection, and a 1:5,000 dilution of mouse monoclonal anti-actin (Abcam) was used as the primary antibody for actin detection. Peroxidase-conjugated goat anti-rabbit (Millipore) and goat anti-mouse (Chemicon) were used as secondary antibodies. Proteins were detected using an ECL Plus Western blotting detection system (GE Healthcare).

**RESULTS**

In initial experiments to monitor and characterize the subcellular localization of Est1p, we utilized strains that overexpress a GFP-tagged variant because the limited abundance of Est1p precludes the use of fluorescence microscopy to examine localization at endogenous levels (24, 28, 29). Importantly, as presented below, we proceeded to determine the functional relevance of Est1p nuclear localization by using an untagged protein expressed from the endogenous EST1 promoter at a low copy number.

**An Est1-GFP fusion protein localizes to the nucleus.** Est1p localizes to the nucleus when expressed from a galactose-inducible promoter (30, 31), suggesting that Est1p possesses an autonomous mechanism for nuclear import. To confirm these results and to identify residues that mediate nuclear localization, an EST1-GFP fusion construct under the control of the inducible GAL1 promoter was cloned into a high-copy-number vector and transformed into cells possessing the dsRED-HDELp fusion (42), a marker of the nuclear envelope. Upon galactose induction, the Est1-GFP fusion protein (Est1-GFPp) localized within the area outlined by dsRED-HDELp and colocalized with Hoechst 33342 staining (Fig. 1A). Est1-GFPp exhibited diffuse fluorescence throughout the nucleus, with a single bright focus within the nuclear envelope but outside the region staining for DNA. This phenotype is consistent with nucleolar localization, as previously reported (30), since the nucleolus resists staining by Hoechst 33342 (43). We concluded that the Est1-GFP fusion protein utilized in this study localizes to the nucleus, with a tendency to concentrate in the nucleolar compartment.

To estimate the extent of overproduction of Est1-GFPp, the expression level upon galactose induction was compared to expression of the same protein from the native EST1 promoter on a centromere vector (Est1-GFPpCEN). While Est1-GFPpCEN was undetectable by Western blotting (Fig. 1B, lane 2), the overexpressed protein was visible when whole-cell extract was diluted up to 81-fold (Fig. 1B, lane 9), placing a lower boundary on the extent of overexpression relative to that of Est1-GFPpCEN. We cannot rule out the possibility that steady-state levels of Est1-GFPpCEN are lower than those of endogenous Est1p. However, the ability of Est1-GFPpCEN to support telomere maintenance (see below) demonstrates that the fusion protein is expressed and at least partially functional. Since there are fewer than 100 molecules of each of the known core components of telomerase in the cell (24, 44), we concluded that galactose-induced Est1-GFPp is expressed in great excess relative to the levels of endogenous telomerase components.

To test Est1-GFPp function, we examined its ability to complement the deletion of EST1. As expected, transformation of the est1Δ strain with an empty vector led to senescence, followed by the appearance of rare survivors using a recombination-based mode of telomere maintenance (45, 46). This phenotype was evi-
localized to the nucleus when overexpressed. (A) Localization of overexpressed Est1-GFP fusion protein. Yeast cells containing pCH101 (2μ EST1-GFP) were grown in galactose-containing medium and examined by live-cell fluorescence microscopy. Hoechst 3342 stains DNA, and the dsRED-HDEL fusion marks the nuclear envelope. Ninety-six percent of GFP-fluorescing cells demonstrated exclusive nuclear localization of Est1-GFPp (merge). Bars = 2 μm. (B) Relative expression levels of the Est1-GFP fusion protein. Whole-cell extracts (WCE) prepared from wild-type cells expressing EST1 from a centromere vector (pRS416-EST1; lane 1) or EST1-GFP from a low-copy-number (pCH101; lane 2) or high-copy-number (pCH109; lanes 3 through 9) vector were separated by gel electrophoresis, Western blotted, and probed with anti-GFP and anti-actin antibodies. Uninduced samples (lanes 3 and 4) were WCE prepared from cells grown in raffinose; induced samples (lanes 5 through 9) were grown in galactose. The fold dilutions of each sample of WCE are indicated. *, nonspecific band. (C) Telomere length analysis of est1Δ cells expressing empty vector (pRS416; lanes 1 and 2) or wild-type EST1 (pRS416-EST1; lanes 2 to 5). The indicated constructs were transformed into an est1Δ strain following loss of a complementing plasmid, and cells were grown for ~100 generations. Marker sizes are indicated in kilobases (kb). Arrows point to bands resulting from amplification of subtelomeric Y′ elements. (D) Telomere length analysis of cells expressing Est1-GFPp from a low- or high-copy-number vector. Constructs described for panel B were transformed into an est1Δ strain, and cells were grown for ~100 generations on solid media containing glucose (lanes 1 to 12) or galactose (lanes 13 to 16). Strains were grown to saturation in the appropriate liquid medium, and genomic DNA was isolated and Southern blotted. Four independent colonies were analyzed for each strain. Marker sizes are indicated in kilobases (kb). Quantification of the Southern blot results is shown to the right of the gel. Error bars represent standard deviations.

To identify specific residues required for nuclear localization, several constructs harboring wild-type EST1 (Fig. 1C, lanes 3 to 5). In contrast to the phenotypes observed for cells harboring the empty vector, both low-level expression and overexpression of the Est1-GFP fusion protein in an est1Δ strain supported normal growth. Expression of the fusion protein from a centromere plasmid resulted in a short but stable telomere length, while cells overexpressing the fusion protein maintained telomeres at a length comparable to that in cells harboring untagged EST1 (Fig. 1D). Thus, the Est1-GFP fusion protein retained functionality, and its overexpression was compatible with normal telomere maintenance.

Three separable regions of Est1p are able to mediate nuclear localization. To identify sequences capable of supporting nuclear import, Est1p was initially subdivided into three regions of 200 to 300 residues. To prevent passive import through the nuclear pore (47), each peptide was expressed as a fusion with two tandem GFP monomers (2GFP) under the control of the inducible GAL1 promoter. The localization phenotypes observed for all 100 GFP-expressing cells were quantified and categorized as exclusively nuclear or cytoplasmic fluorescence (N or C, respectively) or both nuclear and cytoplasmic fluorescence (I). We interpret the I category as representing a partial phenotype in which nuclear localization can occur but is incomplete. Proteins that exhibit localization in the N and I categories, with few or no cells displaying the C phenotype, are considered to be capable of nuclear localization, while those with 70% or more of the cells in the C category are defined as lacking the ability to localize to the nucleus. A few constructs exhibited various levels of vacuolar fluorescence (V) in addition to nuclear and/or cytoplasmic fluorescence. Because Est1p does not appear to possess a vacuolar targeting sequence and the wild-type Est1-GFP fusion protein was not observed in the vacuole (Fig. 1A), such localization was likely artifactual.

As expected, 2GFP alone localized primarily to the cytoplasm, while a fusion between TAgNLS and 2GFP localized primarily to the nucleus (Fig. 2A). Proteins containing the N-terminal 200 amino acids (aa) (NT200) or the central 300 aa (Mid300; residues 199 to 499) of Est1p fused to 2GFP showed either complete or partial nuclear localization in most cells, similar to the localization observed with the Tanl-NLS-GFP fusion. However, fusion of the C-terminal 200 aa (CT200) of Est1p to 2GFP resulted in primarily cytoplasmic localization (Fig. 2A). To lend additional support to the observation that Est1p possesses at least two separable regions that can direct nuclear localization, a region containing the C-terminal 300 aa (CT300) of Est1p was expressed in the context of the 2GFP fusion protein; this fusion also demonstrated the ability to localize to the nucleus (Fig. 2B).

Subdivision of the Mid300 region (aa 199 to 499) revealed that two shorter regions (aa 199 to 350 and 351 to 499) were each able to direct nuclear localization of 2GFP (Fig. 2B). Finally, the region of aa 351 to 499 was divided to produce fragments of aa 351 to 435 and 436 to 499. Only the second of these fragments was consistently observed in the nucleus (Fig. 2B). Each fusion protein was expressed and was of the expected molecular size (see Fig. S1 in the supplemental material). As summarized in Fig. 2C, we concluded that at least three separable regions within the N-terminal 500 aa of Est1p are able to support nuclear localization of 2GFP.

Est1p contains three NLSs that contribute to nuclear localization. To identify specific residues required for nuclear localization, putative NLSs within the three target regions were identified using online NLS prediction programs (PSORT [48, 49], Predict-NLS [50], and cNLS mapper [51, 52]) or through the presence of three or more adjacent basic residues. Positively charged amino...
acids within each candidate NLS were mutated to alanine, and localization was examined in comparison with the appropriate unmutated 2GFP fusion construct. Mutation of lysine 113 or lysines 122 and 123 (positions based on full-length Est1p sequence) in the context of NT200 slightly reduced nuclear localization (Fig. 3A, compare top and middle panels). However, simultaneous mutation of all three lysines (est1-mut1) abrogated nuclear localization (Fig. 3A, bottom panel), suggesting that the N-terminal 200 aa of Est1p contain a bipartite NLS, defined as an NLS that contains two required clusters of positively charged amino acids separated by a short linker sequence (53, 54).

Alanine mutations of two distinct basic clusters lying within the Mid300 region of Est1p (residues 291 to 293 [est1-mut2] and 455 to 458 [est1-mut3]) modestly reduced nuclear localization when mutated separately (Fig. 3B, compare top and middle panels). However, simultaneous mutation of these clusters caused a loss of Mid300 nuclear localization (Fig. 3B, bottom panel). Expression of est1-mut2 in the context of residues 199 to 350 or of est1-mut3 in the context of residues 351 to 499 severely perturbed nuclear localization of the corresponding 2GFP fusion proteins (Fig. 3C). Mutation of two other basic clusters, located between residues 382 and 392, had no effect on localization of the fragment of aa 351 to 499 (Fig. 3D), consistent with our observation that residues 351 to 435 do not mediate nuclear localization (Fig. 2B and C). We concluded that each of the three regions of Est1p shown to independently facilitate nuclear localization contains a single cluster of basic residues (defined by the mut1, mut2, and mut3 mutations) required for localization.

**FIG 2** Three separable regions of Est1p support nuclear localization. (A) Localization analysis of three regions of Est1p. Wild-type cells harboring 2GFP fusions of the indicated proteins under the control of a galactose-inducible promoter in a high-copy-number vector (from top to bottom: pCH200, pCH201, pCH202, pCH206, and pCH210) were grown in galactose-containing medium. Cells were stained with Hoechst 33342 and visualized by live-cell fluorescence microscopy. Adjacent to each set of images is a graph indicating the localization phenotype observed in the GFP-expressing cells. N, nuclear fluorescence only; I, intermediate phenotype (fluorescence in both the nucleus and the cytoplasm); C, cytoplasmic fluorescence only; V, vacuolar fluorescence. N, I, and C are mutually exclusive categories, while any cell exhibiting vacuolar staining was counted in the V category, regardless of other localization observed. n ≥ 100 GFP-expressing cells for each sample. Representative images were selected from at least 3 biological replicates. Bar = 2 μm. See panel C for the location of each construct. (B) Additional mapping of Est1p sequences sufficient to mediate nuclear localization. Experiments were conducted as described for panel A on cells containing plasmids (from top to bottom) pCH211, pCH212, pCH214, pCH218, and pCH219. For the bottom panel, the location of the nucleus was determined by dsRED-HDELp fluorescence. (C) Summary of the regions of Est1p sufficient for nuclear localization, based on the data in panels A and B. Black bars, predominantly nuclear distribution; gray bars, predominantly cytoplasmic distribution.
Mutational analysis reveals the locations of three NLSs within Est1p. (A to D) Live-cell fluorescence microscopy images were generated and quantified as described in the legend to Fig. 2 for cells containing the indicated fusion constructs. The fragment of Est1p analyzed is diagrammed, with mutated residues shown in red. n ≥ 100 GFP-expressing cells for each sample. Bar = 2 μm. Representative images were selected from at least 3 biological replicates for panels A to C. WT, wild type. (A) Mutational analysis conducted in the context of the EST1(NT200)-2GFP fusion (plasmids, from top to bottom: pCH202, pCH203, pCH204, and pCH205). The est1-mut1 allele contains the K113A, K122A, and K123A mutations. (B) Mutational analysis conducted in the context of the EST1(Mid300)-2GFP fusion (plasmids, from top to bottom: pCH206, pCH207, pCH208, and pCH209). The est1-mut2 allele contains the R291A, R292A, and R293A mutations, and the est1-mut3 allele contains the R455A, R457A, and K458A mutations. (C) Analysis of cells expressing mut2 or mut3 Est1p variants in the context of EST1(199–350)-2GFP or EST1(351–499)-2GFP, respectively (plasmids, from top to bottom: pCH212, pCH213, pCH214, and pCH215). (D) Mutational analysis conducted in the context of the EST1(351–499)-2GFP fusion (plasmids, from top to bottom: pCH214, pCH216, and pCH217).

FIG 3
The est1-mut1, est1-mut2, and est1-mut3 mutations were simultaneously introduced into the full-length Est1p-GFP overexpression plasmid utilized for the experiments in Fig. 1. As predicted, the NLS triple mutant [est1-mut1,2,3(FL)] caused cytoplasmic localization (Fig. 4A, middle panel). To determine whether loss of nuclear localization was due solely to loss of NLS function, the TAg-NLS was fused with est1-mut1,2,3(FL) in the context of the GFP overexpression plasmid. The TAg-NLS–est1-mut1,2,3(FL)–GFP fusion protein regained some ability to enter the nucleus, the rescue of mislocalization was incomplete (Fig. 4A, bottom panel).

To investigate redundancy among the three NLSs, the localization phenotypes of the individual est1-mut1, est1-mut2, and est1-mut3 alleles were examined in the context of full-length EST1. The est1-mut1 allele caused a partial reduction in the nuclear localization of Est1p, a phenotype that was completely rescued by fusion with the TAg-NLS (Fig. 4B). This partial phenotype suggests that the first NLS contributes to the nuclear localization of Est1p but that the two remaining NLSs have some ability to direct nuclear localization in its absence. Similar to est1-mut1, the est1-mut2 and est1-mut3 mutations partially perturbed nuclear localization of full-length Est1p (Fig. 4B). However, for reasons that are unclear, fusion of the TAg-NLS only modestly suppressed the localization defect (Fig. 4B).

Autonomous nuclear localization of Est1p contributes to telomere maintenance. To ascertain whether the NLSs contribute to telomere maintenance in vivo, the complementation phenotypes of the individual NLS mutant alleles were examined in the context of full-length, untagged EST1 expressed from the native EST1 promoter in a low-copy-number vector. When the est1-mut1(FL) allele was expressed from a centromere vector in an est1Δ strain, telomeres shortened by an average of 63 bp compared to those in cells harboring the EST1 construct (Fig. 5A). To test whether this decrease in telomere length was due to mislocalization, we fused the TAg-NLS to the N-terminal coding regions of wild-type and mutant EST1. Addition of TAg-NLS to wild-type EST1 caused a small but reproducible increase in telomere length. Importantly, cells expressing the TAg-NLS–est1-mut1 allele maintained telomeres that were only 26 ± 11 bp shorter than those in cells expressing the TAg-NLS–EST1 allele, a decrease in length significantly smaller than the 63 ± 15-bp difference observed between the EST1 and est1-mut1 strains. The ability of the TAg-NLS to substantially rescue the telomere length defect of the est1-mut1 allele is consistent with a functional role for the autonomous localization of Est1p during telomerase biogenesis.

Like est1-mut1, the est1-mut2 and est1-mut3 alleles caused the telomeres to be maintained at a shorter but stable length (Fig. 5B and C) (average decreases of 30 ± 11 bp and 90 ± 7 bp, respectively, relative to the length with EST1). However, in neither case did fusion of the TAg-NLS significantly restore telomere length (average decreases of the TAg-NLS–fused mutant alleles relative to TAg-NLS–EST1 of 33 ± 16 bp and 99 ± 12 bp, respectively), consistent with the lack of rescue observed for the overexpressed proteins (Fig. 4B).

We attempted to test the essential nature of Est1p nuclear localization by expressing an untagged allele containing all three mutations [est1-mut1,2,3(FL)] from a centromere vector. This strain maintained very short telomeres and showed evidence of
subtelomeric Y+ amplification (Fig. 5D), a phenotype observed when telomerase function is lost and rare survivors arise that utilize recombination to maintain viability (Fig. 1C) (45, 46). Although fusion of the TAgNLS with the est1-mut1,2,3 (FL) variant restored some nuclear localization to the GFP-tagged protein (Fig. 4A), addition of the TAgNLS to the untagged est1-mut1,2,3 (FL) low-copy-number construct was unable to restore telomere maintenance in an est1/H9004 strain (Fig. 5D). Thus, the telomere length defect of the est1-mut1,2,3 (FL) allele cannot be attributed solely to a defect in nuclear localization.

Taken together, these data suggest that the three NLS sequences in Est1p contribute in a partially redundant manner to the nuclear localization of Est1p. Rescue of the telomere length defect of the est1-mut1 allele by addition of the TAgNLS demonstrates that normal localization of Est1p is important for telomerase function. However, the mutations required to eliminate the function of the other two NLSs (est1-mut2 and est1-mut3) have additional effects that preclude the unambiguous determination of whether the ability of Est1p to mediate its own nuclear localization is essential for telomerase function.

**Est1p does not require Kap122p or Mtr10p for nuclear import.** The β importins Mtr10p and Kap122p have been implicated in the nuclear import of TLC1 RNA (26, 27, 55, 56). However, the nucleocytoplasmic shuttling of the protein components of telomerase was not explicitly examined. Since our overexpression analysis suggests that Est1p does not require interaction with the other components of telomerase for nuclear localization, we sought to determine whether Est1p also requires MTR10 and

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**FIG 5** TAgNLS rescues telomere shortening of the N-terminal NLS mutant. (A to D) Untagged and TAgNLS fusions of wild-type EST1 or est1-NLS mutants were expressed on a centromere vector under the control of the endogenous EST1 promoter and transformed into an est1/H9004 strain (YKF901). After growth for ~100 generations, genomic DNA was isolated from each strain and Southern blotted (see Materials and Methods). Where appropriate, quantification of the Southern blot results is shown to the right of each gel. After correcting for the telomere lengthening observed in the TAgNLS-EST1 strain (see the text), statistical analysis was performed by one-way ANOVA with Tukey’s honest significant difference (HSD). Marker sizes are indicated in kilobases (kb). Error bars represent standard deviations. (A) Telomere length analysis of est1-mut1(FL). Four independent colonies were analyzed for each strain (pRS416-EST1, lanes 1 to 4; pCH003, lanes 5 to 8; pCH010, lanes 9 to 12; and pCH011, lanes 13 to 16). *, telomere lengths of cells expressing est1-mut1(FL) were significantly shorter than those of the EST1- or TAgNLS-est1-mut1(FL)-expressing strains (P = 0.006). (B) Telomere length analysis of est1-mut2(FL). Six independent colonies were analyzed for each strain (pRS416-EST1, lanes 1 to 6; pCH004, lanes 7 to 12; pCH010, lanes 13 to 18; and pCH012, lanes 19 to 24). There was no statistical difference between telomere lengths of cells harboring est1-mut2(FL) or TAgNLS-est1-mut2(FL) (P = 0.21). (C) Telomere length analysis of est1-mut3(FL). Four independent colonies were analyzed for each strain (pRS416-EST1, lanes 1 to 4; pCH005, lanes 5 to 8; pCH010, lanes 9 to 12; and pCH013, lanes 13 to 16). There was no statistical difference between telomere lengths of cells harboring est1-mut3(FL) or TAgNLS-est1-mut3(FL) (P = 0.54). (D) Telomere length analysis of est1-mut1,2,3(FL). Four independent colonies were analyzed for each strain (pRS416-EST1, lanes 1 to 4; pCH009, lanes 5 to 8; pCH010, lanes 9 to 12; and pCH014, lanes 13 to 16). Arrows point to Y+ amplification, indicative of a failure to complement the est1Δ phenotype (see Fig. 1C). Because the cells utilized recombination to maintain telomeres, telomere length was not quantified.
KAP122 for nuclear accumulation. A kap122Δ strain and a strain harboring a conditional allele of MTR10, mtr10-7, were transformed with the EST1-GFP overexpression construct and with GFP fusions to Nab2p, Rnr4p, and Hrb1p, proteins that depend upon the importins Kap104p, Kap122p, and Mtr10p, respectively, for nuclear import (57–60). As expected, each of these GFP fusions localized primarily to the nucleus in wild-type yeast cells (Fig. 6A).

Even in the complete absence of Kap122p, Est1-GFPp retained nuclear localization (Fig. 6B). The Rnr4-GFP fusion exhibited partial mislocalization (the previously reported phenotype [60]), while Nab2-GFPp localized to the nucleus as expected (Fig. 6B). At the permissive temperature of 18°C, all the GFP constructs showed nuclear localization in mtr10-7 cells. Upon a shift to the restrictive temperature, the Hrb1-GFP fusion protein was redistributed to the cytoplasm as expected, while Est1-GFPp and the

### Table: Protein Localization

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### Figure 6

The β importins Kap122p and Mtr10p are not required for Est1p nuclear localization. (A) Analysis of protein localization in wild-type cells. Live-cell fluorescence microscopy was performed with wild-type cells (BY4741) expressing GFP fusions of NAB2 (pAC719), HRB1 (pHK337), RNR4 (pMH1326), and EST1 (pCH101). Nab2 and Hrb1 were expressed under the control of their native promoters, while RNR4 and EST1 were expressed under the control of galactose-inducible promoters. Quantification for panels A to D was performed as described in the legend to Fig. 2. Representative images were selected from at least 3 biological replicates. Bar = 2 μm. (B) Analysis of protein localization in kap122Δ cells. Live-cell fluorescence microscopy was performed with a BY4741 kap122Δ strain containing the NAB2, RNR4, and EST1 vectors described for panel A. (C) Analysis of protein localization in mtr10-7 cells. Cells containing a temperature-sensitive allele of MTR10, mtr10-7, were transformed with the indicated GFP fusion constructs (described for panel A). Cells were grown to mid-log phase at the permissive temperature of 18°C in selective medium, and galactose was added to cultures containing the RNR4- or EST1-GFP fusion plasmid to induce protein expression. Cultures were split and incubated at 18°C or 37°C. Cells were fixed and visualized as described in Materials and Methods. Black bars, localization at 18°C; gray bars, localization at 37°C. (D) Analysis of protein localization in mtr10-7 kap122Δ cells. The indicated GFP fusion constructs were transformed into an mtr10-7 kap122Δ strain and analyzed as described for panel C.
other control proteins retained nuclear localization (Fig. 6C). To rule out redundancy, localization was examined in mtr10-7 kap122Δ double mutant cells. Once again, the Est1-GFP and Nab2-GFP fusion proteins remained localized to the nucleus at both temperatures tested, while Rnr4-GFPp exhibited a primarily cytoplasmic distribution and Hrb1-GFPp lost nuclear localization after the shift to the restrictive temperature (Fig. 6D). Since Est1p localization was unaffected by perturbations in both KAP122 and MTR10, these results indicate that under conditions of overexpression, Est1p is imported to the nucleus via a different pathway than that of TLC1 RNA.

**Est1p requires the classical nuclear import machinery for import into the nucleus.** The classical nuclear import pathway—defined by binding of the adapter, importin α, to a cargo protein, followed by recruitment of importin β to permit active transport through the nuclear pore (61–63)—is purported to participate in the transport of ~40% of nuclear proteins (64). To test whether the classical nuclear import machinery is required for Est1p nuclear import, localization phenotypes of Est1-GFP, Nab2-GFP, and T$_{αN}$NLS-2GFP fusion proteins were examined in a strain containing a temperature-sensitive allele of yeast importin α, i.e., srp1-54 (65, 66).

At the permissive temperature (25°C), each GFP fusion protein localized predominantly to the nucleus. As expected, Nab2-GFPp, whose nuclear import does not require importin α, retained nuclear localization after incubation at the restrictive temperature (Fig. 7A). However, T$_{αN}$NLS-2GFPp—known to utilize importin α (51)—and Est1-GFPp relocalized to the cytoplasm upon the shift to 37°C, indicating that Est1p requires importin α for nuclear import (Fig. 7A). To rule out the possibility of a nonspecific effect of temperature on Est1-GFPp localization, the Nab2NLS (36, 67) was fused to Est1-GFPp, and localization was monitored in the srp1-54 strain. The Nab2NLS-Est1-GFP fusion protein retained nuclear localization at both permissive and nonpermissive temperatures (Fig. 7A), indicating that mislocalization of Est1-GFPp at the restrictive temperature is due specifically to reduced importin α function.

To exclude the possibility that other nuclear import proteins function in Est1p localization, the EST1-GFP overexpression construct was transformed into a panel of yeast importin mutants. Est1p nuclear import was retained in all strains except those associated with the classical nuclear import machinery, namely, mutants of srp1 and rsl1, encoding the yeast homolog of importin β (Fig. 7B) (65, 68).

**Import of Est1p via the classical pathway contributes to telomere length maintenance.** The results presented above indicate that Est1p requires the SRP1/RSL1 pathway for nuclear import upon overexpression. To address whether this import pathway affects telomere maintenance under endogenous conditions, we examined telomere length in the srp1-54 strain. At the permissive temperature of 25°C, telomere lengths were identical in srp1-54 cells containing either an empty vector or a complementing gene on a centromere vector. After ~100 generations of growth at the semipermissive temperature of 35°C, telomeres were shortened in both the complemented and noncomplemented strains. However, there was a significantly greater decrease in telomere length in cells transformed with the empty vector than in those complemented with wild-type SRP1 (Fig. 8A). Since steady-state telomere length decreases upon growth at an elevated temperature in wild-type yeast strains (69), these data suggest that the exaggerated decrease in telomere length that occurred in the noncomplemented strain was specifically attributable to decreased SRP1 function.

Given the importance of the importin α pathway for the nuclear localization of overexpressed Est1-GFPp, we hypothesized that compromised nuclear localization of endogenous Est1p was responsible for the difference in telomere length between the complemented and noncomplemented srp1-54 strains at the semiper-
FIG 8 Introduction of EST1 into srp1 cells specifically suppresses the telomere length defect observed at high temperature. In panels A, B, D, and E, four independent colonies of each yeast strain were restreaked four times on solid medium and grown to saturation in liquid culture for a total of 100 generations of growth at the indicated temperature. Genomic DNA was isolated, and telomeres were detected by Southern blotting. Error bars represent standard deviations.

(A) Telomere length analysis of strain ACY1563 transformed with an empty vector (VO; pRS413) or a plasmid expressing wild-type SRP1 (CEN; pCH017) and grown at 25°C (lanes 1 to 8) or 35°C (lanes 9 to 16). At 25°C, there was no significant difference in the telomere lengths of cells expressing the empty vector and those expressing SRP1 (P = 0.16 by Student’s t test). However, cells harboring the empty vector had significantly shorter telomeres than those harboring SRP1 when grown at 35°C, by Student’s t test (P = 0.0052).

(B) Telomere length analysis of srp1-54 cells harboring an additional copy of EST1 at 35°C. The srp1-54 strain (ACY1563) was transformed with an empty vector (VO; pRS413) or with centromere vectors containing SRP1 (pCH017), EST1 (pCH016), or Nab2NLS-EST1 (pCH018), each expressed from the native SRP1 or EST1 promoter. Cells containing the SRP1, EST1, or Nab2NLS-EST1 plasmid had significantly longer telomeres than cells containing the empty vector (P = 0.0004, P = 0.007, and P = 0.0004, respectively, by one-way ANOVA with Tukey’s HSD).

(C) The experiment shown in panel B was repeated by growth of an additional eight colonies of the srp1-54 strain, harboring the empty vector or a low-copy-number plasmid expressing SRP1 or EST1 at 35°C. Genomic DNA was isolated, and telomeres were detected by ligation-mediated telomere PCR using a primer specific to the Y′ element (see Materials and Methods). Representative results from four independent colonies of each strain are shown. Telomere lengths were quantified as described in Materials and Methods and are shown in the accompanying graph. Error bars represent standard deviations. Telomeres were significantly longer in strains containing an additional copy of EST1 or SRP1 than in cells containing the empty vector (P < 0.0001 for each by one-way ANOVA with Tukey’s HSD; n = 8).

(D) Telomere length analysis of the srp1-54 strain (ACY1563) complemented by plasmid-borne wild-type SRP1 (CEN; pCH017)—denoted pCENSRP1—and containing either an empty vector (VO; pRS416) or EST1 expressed from a centromere vector (pRS416-EST1) at 25°C. Expression of an additional copy of EST1 did not significantly increase telomere length in the SRP1 background (P = 0.5 by one-way ANOVA).

(E) Telomere length analysis of srp1-54 cells harboring an additional copy of TLC1 RNA expressed from a low- or high-copy-number vector. The experiment was conducted as described for panel B, except that the srp1-54 strain was transformed with constructs containing TLC1 expressed from its native promoter in a centromere (pCH019) or high-copy-number (2μ; pCH020) vector. While expression of SRP1 complemented the telomere length defect of the mutant (P < 0.0001 by one-way ANOVA with Tukey’s HSD), there was no statistical difference between telomere lengths of cells containing the empty vector and those of cells transformed with either TLC1 construct (P = 0.8 for cells harboring pCH018 and P = 0.9 for cells harboring pCH019 by one-way ANOVA with Tukey’s HSD).
missile temperature. If this was true, we predicted that expression of a Nab2NLS-EST1 fusion protein, which attains nuclear localization in the srp1-54 mutant at 37°C when overexpressed, would be sufficient to rescue the telomere length defect at 35°C, even if the protein was expressed at more moderate levels. As shown in Fig. 8B, expression of Nab2NLS-EST1 from a centromere vector rescued telomere length in the srp1-54 strain, to an extent similar to that in the SRP1-complementing plasmid.

Since we were limited to performing these experiments at a semipermissive temperature, we reasoned that slight overexpression of even wild-type EST1 may be sufficient to overcome the telomere shortening observed in the srp1-54 strain at 35°C. When the srp1-54 strain containing either the empty vector or low-copy-number EST1 was grown at the semipermissive temperature, cells expressing additional EST1 had significantly longer telomeres than those harboring the empty vector (Fig. 8B). The decrease in telomere length attributable to reduced Srp1p function at 35°C averaged 57 ± 14 bp, while expression of an extra copy of EST1 restored telomere length by an average of 40 ± 16 bp, as measured by Southern blotting (Fig. 8B).

We confirmed this result by using a different method to measure the telomere lengths for eight additional colonies of each genotype. Amplification of a subset of Y′ telomeres by ligation-mediated PCR to measure the length of the double-stranded telomere sequence confirmed that low-level expression of EST1 rescued the telomere length defect conferred by the srp1-54 allele at 35°C (Fig. 8C). Although the absolute telomere lengths measured by PCR were slightly longer than those measured by Southern blotting (perhaps reflecting extrapolation of migration distances between the 500- and 1,000-bp markers on the Southern blot), the increases in telomere length conferred by EST1 expression relative to empty vector expression were indistinguishable in the two assays (40 ± 16 bp by Southern blotting and 42 ± 16 bp by ligation-mediated PCR).

To confirm that this level of EST1 overexpression does not result in telomere elongation in a wild-type strain, we examined telomere lengths in srp1-54 strains complemented by plasmid-borne SRP1 and additionally expressing either low-copy-number EST1 or an empty vector at the permissive temperature. Under these conditions, an additional copy of EST1 did not affect telomere length (Fig. 8D). Furthermore, the rescue of telomere length by additional EST1 expression was specific, since it was not observed upon introduction of TLC1 RNA, at low or high expression levels, into the srp1-54 strain (Fig. 8E). We concluded that the telomere attrition observed in the importin α mutant at the semipermissive temperature was substantially due to Est1p mislocalization, indicating that the autonomous localization of Est1p to the nucleus via the classical nuclear import pathway contributes to normal telomere length maintenance.

DISCUSSION

Here we describe the first in-depth characterization of the mechanism through which a protein component of S. cerevisiae telomerase undergoes nuclear localization. While our studies, of necessity, used an overexpression approach to characterize sequences required for Est1p nuclear localization, we established the functional relevance of these sequences by showing that telomere shortening occurs when the cis-acting sequences (NLSs) or trans-acting import machinery (importins α and β) is mutated. Furthermore, this telomere maintenance defect is specific, since it can be rescued by conditions predicted to restore nuclear localization of Est1p.

Our overexpression studies demonstrate that Est1p contains three sequences that mediate nuclear localization and that mutation of any one NLS within full-length Est1p only partially affects the exclusive nuclear localization of the protein (Fig. 4). Such redundancy is not unprecedented, and many yeast proteins that contain multiple NLSs—including ribosomal proteins and a subset of the MCM proteins (70–72)—are part of multiprotein complexes. At endogenous expression levels, we have been able to unambiguously demonstrate a contribution to telomere length maintenance by only the most N-terminal NLS, since mutations in the second and third NLSs (est1-mut2 and est1-mut3) affect protein function(s) in addition to localization. Therefore, the N-terminal NLS may contribute disproportionately to Est1p nuclear localization under endogenous conditions.

To determine whether Est1p NLSs contribute to telomerase function, we examined the consequence of mutating these sequences in the context of full-length Est1p expressed at or near normal levels. Consistent with the partial mislocalization of the est1-mut1(FL)-encoded protein, expression of the est1-mut1 allele in an est1A strain resulted in short but stable telomeres. This defect was suppressed by fusion of the mutant protein with the Tα-NLS (Fig. 5A), indicating that the autonomous nuclear localization of Est1p contributes to normal telomerase function.

We were unable to identify a triple-NLS-mutant allele that is uniquely defective for nuclear import, perhaps reflecting effects of the multiple mutations on Est1p folding and/or function. Thus, although we favor the idea that the NLSs of Est1p are essential for telomere maintenance, we cannot rule out the possibility that additional binding partners partially compensate for the loss of Est1p NLS function when the protein is expressed at endogenous levels.

TLC1 RNA acquires a 2,2,7-trimethylguanosine cap as a step in its maturation to become a functional component of telomerase, and it possesses a binding site for the Sm proteins, thus making telomerase a small nuclear ribonucleoprotein particle (snRNP) (73). Another class of snRNPs with functions vital to the cell include the uridine-rich snRNPs (UsnRNP) that comprise the splicesome (74). Although the mechanisms controlling UsnRNP biogenesis in yeast have yet to be elucidated completely, current data support the assembly of these ribonucleoproteins in the cytoplasm prior to nuclear import of the assembled complex (74). Telomerase biogenesis has been hypothesized to occur in a similar manner, with the most prevalent model asserting that the protein components of the enzyme assemble onto the RNA-like beads on a string before shuttling of the complex into the nucleus (75).

Our demonstration that Est1p nuclear translocation via the classical import pathway is important for normal telomere length maintenance (Fig. 8) suggests that there may be additional complexity to the current model of telomerase biogenesis. TLC1 nuclceotidyltransferase shuttling depends on the nuclear exportin Crm1p and the β importins Mtr10p and Kap122p (26, 27, 76). In contrast, the nuclear import of overexpressed Est1p is unperturbed in mutants of Kap122p and/or Mtr10p (Fig. 6). Nuclear localization of TLC1 is unaffected at the restrictive temperature in an srl1 strain (27), while overexpressed Est1p is excluded from the nucleus under these conditions (Fig. 7). Finally, overexpression of TLC1, but not Est1p, in an mtr10 strain rescues the telomere length defect of the mutant (26). In contrast, telomere shortening
occurs when trafficking through the classical import pathway is disrupted, and this defect is counteracted by expression of excess Est1p, but not TLC1 RNA (Fig. 8). Together, these data point to independent nuclear localization of TLC1 RNA and Est1p.

A possible explanation for these findings is that Est1p does not assemble with the telomerase holoenzyme in the cytoplasm but rather is imported autonomously, associating with telomerase at a later step of biogenesis within the nucleus. This model is consistent with the cell-cycle-regulated abundance of Est1p and with the ability of Est1p to disrupt the nuclear localization of TLC1 RNA (27). However, as previously suggested, it may be the nuclear retention of TLC1 RNA, rather than its import into the nucleus, that is disrupted when EST1 is deleted.

The studies described here address only the mechanism of Est1p import and do not clarify the trafficking of the other protein components of telomerase. A GFP fusion with Est2p also localizes with the cell-cycle-regulated abundance of Est1p and with the later step of biogenesis within the nucleus. This model is consistent with the cell-cycle-regulated abundance of Est1p and with the ability of Est1p to disrupt the nuclear localization of TLC1 RNA (27). However, as previously suggested, it may be the nuclear retention of TLC1 RNA, rather than its import into the nucleus, that is disrupted when EST1 is deleted.

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47. Goldstein AL, McCusker JH.


49. Horton P.


