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A Yeast Telomerase Complex Containing the Est1 Recruitment Protein Is Assembled Early in the Cell Cycle

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Supporting Information

ABSTRACT: In budding yeast, association of the Est1 regulatory protein with telomerase is thought to be limited to the late S phase, when telomere elongation occurs. By monitoring the stoichiometry of telomerase subunits, we show instead that a telomerase complex containing Est1 is assembled much earlier in the cell cycle. We also report a biochemical interaction between Est1 and the telomere binding protein Cdc13 that recapitulates the previously observed genetic relationship between *EST1* and *CDC13*. This supports a model in which regulated binding of Cdc13 to chromosome termini dictates subsequent interaction of a recruitment-competent telomerase complex with telomeres.

In the budding yeast *Saccharomyces cerevisiae*, telomerase is present in very low abundance, at a level that is ∼2-fold lower than the number of chromosome ends[1] during the period of the cell cycle when telomeres are elongated.[2] This disparity implies that a high level of regulation is needed to ensure that underelongated telomeres are preferentially targeted by telomerase.[3] One key regulatory node involves the Est1 regulatory subunit of telomerase that is essential for telomere replication in vivo but dispensable for enzyme catalysis in vitro.[4,5] Two competing models have been proposed for the role of Est1. The first model, driven largely by genetic observations, proposes that recruitment of telomerase to its site of action is driven by an interaction between the telomerase-associated Est1 subunit and the telomere-bound Cdc13 protein.[6−8] A second model, which has primarily depended on chromatin immunoprecipitation, proposes that the catalytic core of telomerase is constitutively bound to telomeres in an inactive state and becomes "activated" in the late S phase through regulated association of Est1 with chromosome termini.[9,10]

Absent from these prior studies has been a direct assessment of the relative stoichiometry of telomerase subunits throughout the cell cycle, as these two models make different predictions regarding the timing of association of Est1 with the telomerase complex. This omission has been caused by the low abundance of telomerase, combined with the difficulty in introducing high-affinity epitope tags that do not impair function.[11,12] We have addressed this using an assay that assesses the relative stoichiometry of the Est1 regulatory subunit with the Est2 catalytic subunit. This relies on a strain in which Est1 and Est2 contain identical $(myc)_{12}$ epitopes as well as a $(FLAG)_3$ epitope on Est2, thereby permitting levels of the two proteins to be simultaneously monitored following anti-FLAG immunoprecipitation (IP). Examination of Est1 and Est2 protein levels in extracts prepared from this strain indicated that Est1 was in excess relative to Est2 throughout most of the cell cycle (Figures S1 and S2 of the Supporting Information). Thus, this strategy targets the limiting factor in the telomerase complex (Est2), which could be quantitatively depleted from extracts by anti-FLAG IP (Figure S1 of the Supporting Information). The epitope-tagged versions of each protein were integrated into the genome in place of the endogenous *EST1* and *EST2* genes, ensuring protein levels comparable to that of a wild-type strain. These modifications also had no effect on telomerase function (in contrast to the behavior of previously constructed strains with epitope tags on telomerase subunits[9,12]), as evidenced by the wild-type telomere length displayed by this strain (Figure 1A).

We recently used this assay to demonstrate that Est1 interacts with the telomerase complex in a 1:1 stoichiometry relative to Est2.[13] This previous experiment monitored telomerase in an asynchronous culture, which might miss transient perturbations in the Est1:Est2 ratio. To address this,

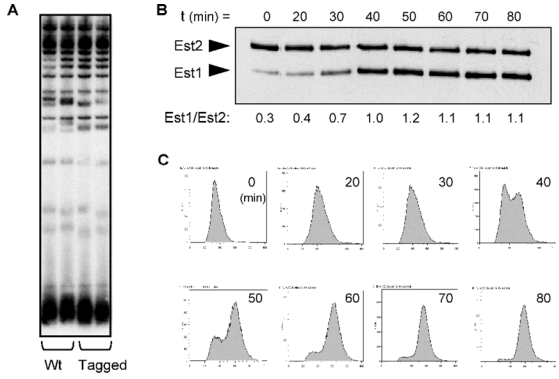

Figure 1. Est1:Est2 stoichiometry through the cell cycle. (A) Telomere length of a wild-type strain (lanes 1 and 2) compared to an Est1-$(myc)_{12}$ $(FLAG)_3$-$(myc)_{12}$-Est2 strain (lanes 3 and 4). (B) An anti-myc Western blot of anti-FLAG immunoprecipitates prepared from cultures released from a G1 phase arrest. (C) FACS analysis of the cultures used for panel B. An independent repeat is shown in Figure S2 of the Supporting Information.

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Est1 and Est2 protein levels in Est2 IPs were examined through the cell cycle. Cells were arrested in the G1 phase by treatment with α-factor; aliquots were collected every 10 min after release into the cell cycle, and anti-FLAG IPs were examined for the presence of the Est1 and Est2 proteins by anti-myc Western blots. In cells arrested in the G1 phase, the level of Est1 in the complex was ~3-fold below that of Est2 (Figure 1B). However, following release into the cell cycle, the amount of Est1 protein in Est2 IPs began to increase. By the second time point after release, the ratio of Est1 to Est2 was already 0.7:1, and by 40 min, when a large percentage of cells were still progressing through the S phase (Figure 1C), the ratio was 1:1, which was maintained as cells continued into the G2/M phaae (Figure 1 and Figure S2 of the Supporting Information). These results show that a telomerase complex containing Est1 and Est2 is assembled early in the S phase, well before the period when telomerase elongates telomeres.[2]

Although Est1 protein levels in extracts were in excess throughout most of the cell cycle, Est1 and Est2 were roughly equivalent in the G1 phase (Figure S2 of the Supporting Information). As pointed out previously,[14] lower Est1 protein levels could account for the reduced amount of Est1 in the telomerase complex at the start of the cell cycle. To test this, a strain was created with a tandem duplication of Est1-$(myc)_{12}$, thereby increasing the dosage of Est1 by 2-fold. When the telomerase stoichiometry from this tandem duplication strain was examined, the ratio of Est1 to Est2 was 1:1 even in Est2 IPs from cells arrested in the G1 phase (Figure 2A). This ratio was

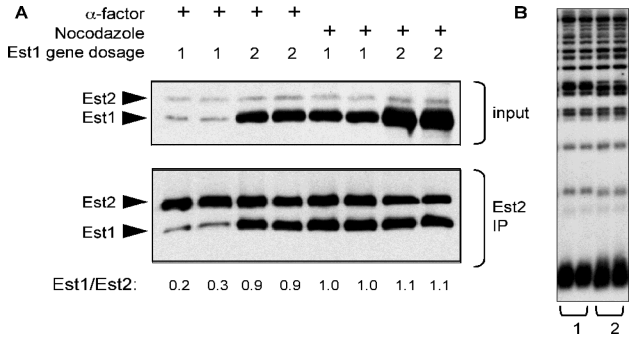

Figure 2. Constitutive association of Est1 with telomerase does not impact telomere length. (A) Anti-myc Western blots of inputs and anti-FLAG IPs from isogenic strains bearing one or two copies of Est1-$(myc)_{12}$, arrested in the G1 (α-factor) or G2/M (nocodazole) phase. (B) Telomere length of wild-type and tandem duplication strains (indicated by 1 or 2, respectively, equivalent to one or two copies of *EST1*).

constant throughout the cell cycle (Figure 2 and Figure S3 of the Supporting Information), indicating that when Est1 protein levels were no longer limiting, the Est1:Est2 ratio in the telomerase complex was continuously maintained at 1:1.

However, even though Est1 was constitutively associated with the complex, there was no impact on telomere length. The effects of a 2-fold increase in Est1 gene dosage on telomere length were examined in the strain bearing the tandem duplication of Est1-$(myc)_{12}$ (Figure 2B) and also in a strain with a tandem duplication of Est1 in an otherwise completely wild-type strain (data not shown). These observations establish that regulated association of Est1 with the telomerase complex does not contribute to telomere homeostasis, in contrast to previous proposals.[9,14]

An alternative source of telomere length regulation stems from the interaction between Est1 and the telomere-bound Cdc13 protein, which led us to propose that Cdc13 recruits telomerase to its site of action by binding the Est1 telomerase subunit.[6,7] This model was further supported by a genetic relationship between *CDC13* and *EST1*, as revealed by the behavior of reciprocal cosuppressing mutations in these genes.[15] Whereas *cdc13-2* and *est1-60* single-mutant strains each exhibit a severe telomere replication defect, telomeres are restored to near wild-type length in the *cdc13-2 est1-60* double-mutant strain.[15] These two alleles are "charge swap" mutations (*cdc13-2* and *est1-60* correspond to E → K and K → E mutations, respectively), indicating that this genetic relationship had uncovered a salt bridge that contributes to the binding between Est1 and Cdc13.

This was recently challenged by an in vitro assessment of the potential interaction between recombinant Est1 and Cdc13 proteins expressed in *Escherichia coli*, as the association between these two proteins was not abolished by mutations in either protein in this in vitro binding assay.[16] However, expression of soluble, nonaggregated versions of full-length Cdc13 and Est1 proteins has been a substantial technical hurdle for multiple laboratories (ref 17 and personal communication of D. Wuttke). Thus, the inability to recapitulate the genetic observations in this prior study[16] might simply reflect an inability to obtain structurally well-behaved recombinant Est1 and Cdc13 proteins from *E. coli*.

This potential caveat prompted us to examine the association between the Est1 and Cdc13 proteins in yeast extracts, using a strain with $(myc)_{12}$ and $(FLAG)_3$ epitopes on Est1 and Cdc13, respectively, with each tagged protein expressed from its endogenous promoter. Following anti-FLAG IP from extracts, an interaction between Cdc13 and Est1 could be detected, albeit at low levels (Figure 3A). The weak binding of Est1 to

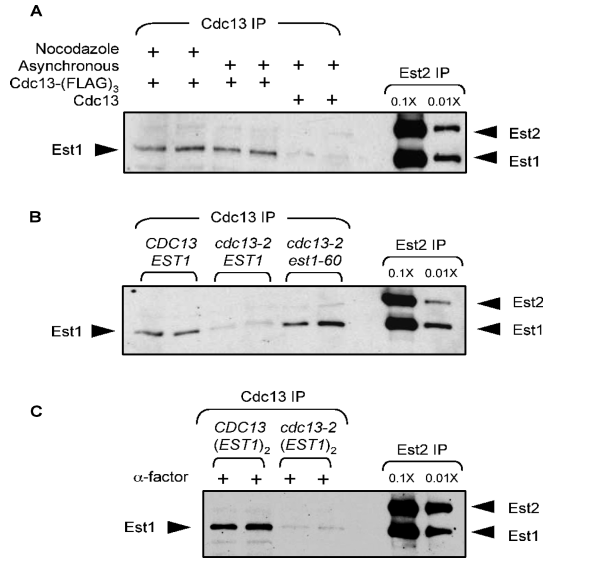

Figure 3. Interaction between Est1 and Cdc13. (A) Anti-myc Western blots of anti-FLAG IPs prepared from strains expressing Cdc13-$(FLAG)_3$ (lanes 1–4), Cdc13 (lanes 5 and 6), or $(FLAG)_3$-$(myc)_{12}$-Est2 (0.1× and 0.01× indicate the amount of IP relative to the amount loaded in lanes 1–6). (B) Same as panel A, but mutations were introduced at the genomic location of the tagged Est1 and Cdc13 proteins (C) Same as panel A, but using *CDC13* and *cdc13-2* versions of the tandem duplication $[Est1\text{-}(myc_{12})]_2$ strain used in Figure 2.

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Cdc13 was consistent with past challenges in observing this interaction from yeast extracts using proteins expressed at endogenous levels.[18] Nevertheless, the signal was clearly above background, as assessed by a control anti-FLAG IP from a strain lacking the $(FLAG)_3$ epitope on Cdc13 (lanes 5 and 6 of Figure 3A). Additional controls further support the specificity of this interaction (Figure S5 of the Supporting Information) and also demonstrate that it is not mediated by a DNA bridge (Figure S6 of the Supporting Information).

The ability to detect binding between Est1 and Cdc13 allowed us to ask about the impact of defects in either protein on this association. As predicted, the recruitment-defective Cdc13-2 mutant protein had lost the ability to IP the Est1 protein (Figure 3B). Strikingly, binding between the mutant Cdc13-2 and Est1-60 proteins was restored to a degree that was comparable to that of the wild-type Cdc13−Est1 interaction. This result was highly reproducible, as it was observed in three independent experiments. These biochemical results therefore recapitulate our prior genetic observations,[15] supporting the conclusion that these two proteins interact in vivo through surfaces defined by the location of these two mutations.

We also exploited the increased levels of Est1 protein in the strain bearing a tandem duplication of *EST1* to ask if the Est1−Cdc13 association could be observed in the G1 phase of the cell cycle. An additional copy of the $(myc)_{12}$-tagged *EST1* gene was introduced into the Cdc13-$(FLAG)_3$ strain, and anti-FLAG IPs from α-factor-arrested cells were examined on anti-myc Western blots. Notably, an interaction was observed between Est1 and Cdc13, in a *cdc13-2*-dependent manner (Figure 3C), in a phase of the cell cycle when telomerase does not elongate telomeres.[2] This argues that the physical interaction between Est1 and Cdc13 is not dependent on cell cycle-specific regulatory event(s) and does not require prior binding of Cdc13 to telomeres.

This study shows that association of the Est1 protein subunit with the catalytic core is not a regulatory event that contributes to telomere homeostasis. In contrast, binding of Cdc13 to telomeres is highly regulated, with association peaking in the late S phase.[9,19] This suggests a simple model in which assembly of a recruitment-competent telomerase complex containing both Est1 and Est2 occurs early in the cell cycle. Subsequent binding of chromosome termini by Cdc13 allows recruitment of the telomerase holoenzyme, through a direct interaction between Cdc13 and the Est1 subunit of the telomerase holoenzyme. These results, which are also supported by live cell imaging of the telomerase RNA in single cells,[20] argue that a primary regulatory event that initiates telomere elongation during the late S phase is the regulated association of Cdc13 with telomeres.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1−S7 and detailed experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Both authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Mozdy, A. D., and Cech, T. R. (2006) *RNA 12*, 1721−1737.
(2) Marcand, S., Brevet, V., Mann, C., and Gilson, E. (2000) *Curr. Biol. 10*, 487−490.
(3) Teixeira, M. T., Arneric, M., Sperisen, P., and Lingner, J. (2004) *Cell 117*, 323−335.
(4) Lundblad, V., and Szostak, J. W. (1989) *Cell 57*, 633−643.
(5) Cohn, M., and Blackburn, E. H. (1995) *Science 269*, 396−400.
(6) Nugent, C. I., Hughes, T. R., Lue, N. F., and Lundblad, V. (1996) *Science 274*, 249−252.
(7) Evans, S. K., and Lundblad, V. (1999) *Science 286*, 117−120.
(8) Bianchi, A., Negrini, S., and Shore, D. (2004) *Mol. Cell 16*, 139−146.
(9) Taggart, A. K., Teng, S. C., and Zakian, V. A. (2002) *Science 297*, 1023−1026.
(10) Chan, A., Boule, J. B., and Zakian, V. A. (2008) *PLoS Genet. 4*, e1000236.
(11) Sabourin, M., Tuzon, C. T., Fisher, T. S., and Zakian, V. A. (2007) *Yeast 24*, 39−45.
(12) Tuzon, C. T., Wu, Y., Chan, A., and Zakian, V. A. (2011) *PLoS Genet. 7*, e1002060.
(13) Lubin, J. W., Tucey, T. M., and Lundblad, V. (2012) *RNA 18*, 1597−1604.
(14) Osterhage, J. L., Talley, J. M., and Friedman, K. L. (2006) *Nat. Struct. Mol. Biol. 13*, 720−728.
(15) Pennock, E., Buckley, K., and Lundblad, V. (2001) *Cell 104*, 387−396.
(16) Wu, Y., and Zakian, V. A. (2011) *Proc. Natl. Acad. Sci. U.S.A. 108*, 20362−20369.
(17) Anderson, E. M., Halsey, W. A., and Wuttke, D. S. (2002) *Nucleic Acids Res. 30*, 4305−4313.
(18) Li, S., Makovets, S., Matsuguchi, T., Blethrow, J. D., Shokat, K. M., and Blackburn, E. H. (2009) *Cell 136*, 50−61.
(19) Takata, H., Kanoh, Y., Gunge, N., Shirahige, K., and Matsuura, A. (2004) *Mol. Cell 14*, 515−522.
(20) Gallardo, F., Laterreur, N., Cusanelli, E., Ouenzar, F., Querido, E., Wellinger, R. J., and Chartrand, P. (2011) *Mol. Cell 44*, 819−827.