

Regulation of Telomere Structure and Functions by Subunits of the INO80 Chromatin Remodeling Complex^{∇†}

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ATP-dependent chromatin remodeling complexes have been implicated in the regulation of transcription, replication, and more recently DNA double-strand break repair. Here we report that the Ies3p subunit of the *Saccharomyces cerevisiae* INO80 chromatin remodeling complex interacts with a conserved tetratricopeptide repeat domain of the telomerase protein Est1p. Deletion of *IES3* and some other subunits of the complex induced telomere elongation and altered telomere position effect. In telomerase-negative mutants, loss of Ies3p delayed the emergence of recombinational survivors and stimulated the formation of extrachromosomal telomeric circles in survivors. Deletion of *IES3* also resulted in heightened levels of telomere-telomere fusions in telomerase-deficient strains. In addition, a delay in survivor formation was observed in an Arp8p-deficient mutant. Because Arp8p is required for the chromatin remodeling activity of the INO80 complex, the complex may promote recombinational telomere maintenance by altering chromatin structure. Consistent with this notion, we observed preferential localization of multiple subunits of the INO80 complex to telomeres. Our results reveal novel functions for a subunit of the telomerase complex and the INO80 chromatin remodeling complex.

Telomeres are natural chromosome ends consisting of a repetitive DNA sequence as well as cognate DNA binding proteins (5, 9). This specialized nucleoprotein complex normally renders the termini inaccessible to degradative and recombinogenic activities. This general inaccessibility is consistent with transcriptional repression of telomere-proximal genes, also known as telomere position effect (TPE) (56). On the other hand, certain telomere transactions, including replication and telomerase-mediated telomere extension, probably entail “opening” of the ends to allow for binding of requisite factors (5, 14). Indeed, evidence for reconfiguration of telomere nucleoprotein structure during the cell cycle is beginning to emerge (50, 52), though the underlying mechanisms are not well understood.

Implicit in the protective role of the telomere complex is its ability to avert recognition of the natural chromosome ends as double-strand breaks (DSBs). At other chromosomal loci, DSBs evoke a DNA damage response that results in pausing of the cell cycle and recruitment of repair proteins, followed by resolution of the broken ends through recombination or non-homologous end joining (59). Thus, telomeres must interact with a set of factors that are distinguishable from those at DSBs. As demonstrated in a variety of studies, alterations in telomere structure due to mutations in either telomere pro-

teins or the underlying sequences can render telomeres recognizable by the DNA damage sensors and repair proteins (32, 33, 53). Another well-studied example of alteration in telomere structure is induced by progressive telomere attrition following multiple rounds of cell division due to incomplete end replication. When telomeres become critically short, they lose the ability to form higher-order structures that are necessary for telomere protection. Instead, the denuded telomere ends come to be recognized as DSBs and a DNA damage response is triggered (16, 21, 27). The structural changes that transpire at the “critically shortened” stage and that are responsible for triggering the DNA damage response are not clearly understood.

As alluded to above, telomere DNA cannot be fully duplicated by the chromosomal replicative polymerases due to the “end replication problem” (38). In unicellular organisms and highly proliferative tissues of multicellular organisms, the end replication problem is averted by a specialized reverse transcriptase known as telomerase. Telomerase is competent at extending the 3′ end of the telomere terminal repeat and consists minimally of two components: an RNA which encodes the telomere repeats and a reverse transcriptase-like protein that utilizes the RNA as a template for telomere synthesis (4, 8, 9, 24, 37). A multitude of telomerase-associated polypeptides have been identified in diverse organisms and implicated in telomerase assembly and functions. While most of these polypeptides are not evolutionarily conserved, an exception is a protein family exemplified by *Saccharomyces cerevisiae* Est1p. Yeast Est1p is absolutely essential for telomere maintenance in vivo but dispensable for telomerase activity in vitro. It has been shown to interact with telomeric DNA, telomerase RNA, and a single-stranded telomere end-binding protein known as Cdc13p (43, 45, 61). The RNA and Cdc13p binding activities of Est1p enable it to mediate the recruitment of the telomerase

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complex to telomere ends (41). Est1p also serves a postrecruitment or activating function, though its mechanism in this capacity is not understood (17, 52). More recently, Est1p homologues in *Candida albicans*, *Schizosaccharomyces pombe*, and humans were identified and implicated in telomere maintenance and protection (3, 44, 49, 51). The most conserved region of Est1p family members is generally located near the N terminus and possesses unmistakable features of an alpha-helical motif known as TPR (*tetratricopeptide repeat*) (49). While present in proteins with diverse functions, each of the well-characterized TPR domains uses a groove formed on its concave surface for binding another peptide (6, 13). Surprisingly, all of the critically important functions of yeast Est1p have previously been mapped to the C terminus of the protein, away from the TPR domain, suggesting that this domain may interact with a novel target(s).

ATP-dependent chromatin remodeling complexes are multisubunit protein machines that use the energy of ATP hydrolysis to move or evict nucleosomes, thereby altering chromatin structure and influencing the accessibility of DNA to other factors (36, 60). Each complex is characterized by a catalytic polypeptide that belongs to the SWI2/SNF2 superfamily of ATPases. This catalytic polypeptide may contain additional motifs, e.g., a SANT domain in the ISWI-related family members. These additional motifs and other subunits of the complex are thought to target the complex to diverse chromosomal locations and modulate its interaction with other chromatin components. Voluminous data implicate ATP-dependent chromatin remodeling complexes in the transcriptional regulation of a large number of genes. However, a possible role(s) for these complexes in the DNA damage response is beginning to emerge. In particular, mutants that are deficient in subunits of the INO80, SWI/SNF, and RSC complex displayed hypersensitivity to genotoxic agents (11, 46, 47). Furthermore, subunits of these complexes are recruited to DSBs, implying that they act directly at damage sites (11, 35, 47, 58). However, no chromatin remodeling complex has hitherto been directly implicated in telomere regulation. In this report, we demonstrate an interaction between Est1p and an INO80 subunit named Ies3p. We also present evidence supporting a role for subunits of the INO80 complex in the regulation of telomere structure and functions in both telomerase-positive and -negative cells.

MATERIALS AND METHODS

Yeast strains and plasmids. The *ino80-Δ*, *arp5-Δ*, and *arp8-Δ* strains have been described previously (46). The *ies3-Δ* strains (in both W303a and BY4741) were generated by homologous recombination using a disruption cassette that contains 500 bp of *IES3* upstream sequences and 500 bp of downstream sequences flanking the *HIS3* marker. The *est1-Δ* and *est2-Δ* strains were similarly derived using a *URA3-* and a *kanMX4*-based cassette, respectively. Two *ies3-Δ est1-Δ* double mutant strains were made by sporulating a heterozygous diploid strain (W303a background). Other *ies3-Δ est1-Δ* and *ies3-Δ est2-Δ* mutants were constructed by deleting the *IES3* gene from the *est1-Δ* and *est2-Δ* strains described above. The *arp8-Δ est1-Δ* double mutant (in BY4741 background) was constructed by sequential disruption of the *EST1* gene (with the *HIS3* marker) and the *ARP8* gene (with the *LEU2* marker). To facilitate analysis of survivor formation, the strain was initially complemented with a *URA3-* and *EST1*-containing plasmid. The phenotypes of the double mutant were analyzed after loss of the plasmid through 5-fluoroorotic acid (5-FOA) selection. The *ies1-Δ*, *ies2-Δ*, *ies4-Δ*, *ies5-Δ*, and *ies6-Δ* strains were purchased from Open Biosystems (Huntsville, AL), and their genotypes were confirmed by PCR. The mutant strains used for TPE analysis are all derivatives of UCC3505 (48) in which the respective

genes have been replaced with the *kanMX4* marker using a PCR-mediated gene disruption strategy.

Telomere length analysis. Southern analysis of telomere restriction fragments was carried out as previously described using genomic DNA that had been digested with either PstI or XhoI (28).

Two-dimensional gel electrophoretic analysis of telomeric DNAs. The two-dimensional gel analysis was performed according to the protocol of Brewer and Fangman (7) as modified by Cohen and Lavi (12). Briefly, the first dimension was run at 0.5 V/cm for 16 h in the absence of ethidium bromide, while the second dimension was run at 5 V/cm for 5 h in the presence of 0.3 μg/ml ethidium bromide. The DNAs in the gels were transferred to a nylon membrane and probed with labeled poly(dG-dT) · poly(dA-dC) as in the case of standard telomere Southern blots.

Telomere-telomere (T-T) fusion analysis. Fusions between telomeres were assayed precisely as described by Mieczkowski et al. (33), except that PCR was performed in an ATC401 thermocycler (CLP USA, San Diego, CA) and the cycle number was increased to 37. The control PCRs for the *HIS4* locus were also performed as described previously (33).

Survivor analysis. A diploid *EST1/est1-Δ IES3/ies3-Δ* strain was sporulated, and several tetrads were dissected to generate several pairs of isogenic *est1-Δ* and *est1-Δ ies3-Δ* clones. The original colonies were streaked once, and the new colonies were used to inoculate 5-ml liquid cultures. After the initial cultures were grown to saturation, they were diluted in fresh 5-ml cultures to a cell density of 2×10^5 cells/ml and grown overnight (20 h), at the end of which the cell density was measured. The dilution and growth were continued for ~10 to 12 days until survivors had emerged for both the *est1-Δ* and *est1-Δ ies3-Δ* clones. For some dilution series, the bulk of the culture from each day was harvested and processed for chromosomal DNA isolation and the resulting DNA samples were subjected to telomere length analysis. For survivor analysis of the *est1-Δ arp8-Δ* double mutant, we started with isogenic *est1-Δ* and *est1-Δ arp8-Δ* clones whose telomeres were initially maintained by plasmid copies of *EST1*. The clones were selected for loss of the plasmid on 5-FOA plates, and the resulting colonies were restreaked once prior to inoculation into 5-ml liquid cultures. The continual liquid subculturing was then performed as described above.

ChIP. Chromatin immunoprecipitation (ChIP) was performed on either the BY4741 strain (not tagged) or BY4741-derived strains bearing FLAG₃-tagged Ies3p, Ino80p, or Nhp10p according to the method of Morrison et al. (35) with some modifications. Cells were fixed with 1% formaldehyde for 20 min at 30°C, and cross-linking was quenched with 125 mM glycine for 5 min at 30°C. Formaldehyde-fixed (cross-linked) or mock-treated (non-cross-linked) cells were lysed by glass beads, and subsequently the lysates were sonicated six times for 5 s (constant duty cycle, 35 to 40% output) to shear DNAs to a mean length of ~600 bp. Agarose gel electrophoresis and ethidium bromide staining were used to confirm the size of sonicated DNA fragments and to normalize the amount of extracts used for IP. Each IP was carried out with 2.5 μg of anti-FLAG M2 antibody (Sigma) in combination with 17.5 μl of Dynabeads protein G (Dyna) at 4°C for 2 h. Ten percent of each cell lysate was set aside and used as input samples. IP samples were washed as described previously (35) and eluted with 0.4 mg/ml of 3× FLAG peptide in radioimmunoprecipitation assay buffer at room temperature for 40 min (35). Cross-linking was reversed at 65°C for 12 h, and DNA was extracted with phenol-chloroform, precipitated with ethanol, and resuspended in 50 μl of Tris-EDTA. In paired PCRs, a nontelomeric control locus (an intergenic site on the left arm of chromosome V [Intergene-V] or a site on chromosome VII [Intergene-VII]) was amplified together with either a telomeric locus on the right arm of chromosome VI (Tel-VI), a telomeric locus on the left arm of chromosome XV (Tel-XV), or the subtelomeric region of the right arm of chromosome VI (Subtel-VI). General PCR conditions were the following: 26 to 28 cycles of 94°C for 30 s, 55 to 57°C for 30 s, and 72°C for 30 s with primer pairs Tel-VI (5'-TTTAAACGGTGATTATTAGGTGGATTTTATATTAGTCTAC-3' and 5'-CATTGCGCCCCATGACCAGTC-3'), Tel-XV (5'-TAACCCGTCCAACCTGTCT-3' and 5'-ATACTATGATCATCCGTGGGC-3'), Subtel-VI (5'-CAAGCTTCCAATATCACGAGTAAGG-3' and 5'-CTGAGACGAAGTCGTTGCTAAAATC-3'), Intergene-V (5'-GGCTGTCAGAATATGGGGCCGTAGTA-3' and 5'-CACCCGGAAGCTGCTTTCACAATAC-3'), and Intergene-VII (5'-CCCACCACCGATAACGACAAG-3' and 5'-CCAACAATGAGGC GGAACC-3'). PCR products were separated on 2.5% agarose gels, ethidium bromide stained, and imaged on a gel documentation system. Signals were quantified using ImageQuant software (Molecular Dynamics Inc.). For calculation of relative enrichment, standard curves for both the telomeric and control loci were generated using the input signals. The amount of IP DNA as a fraction of the input DNA (before amplification) was then derived from the standard curves. Dividing the fraction value obtained for the telomere locus by that for the control locus then gives the enrichment (*n*-fold). For example, if the telomere

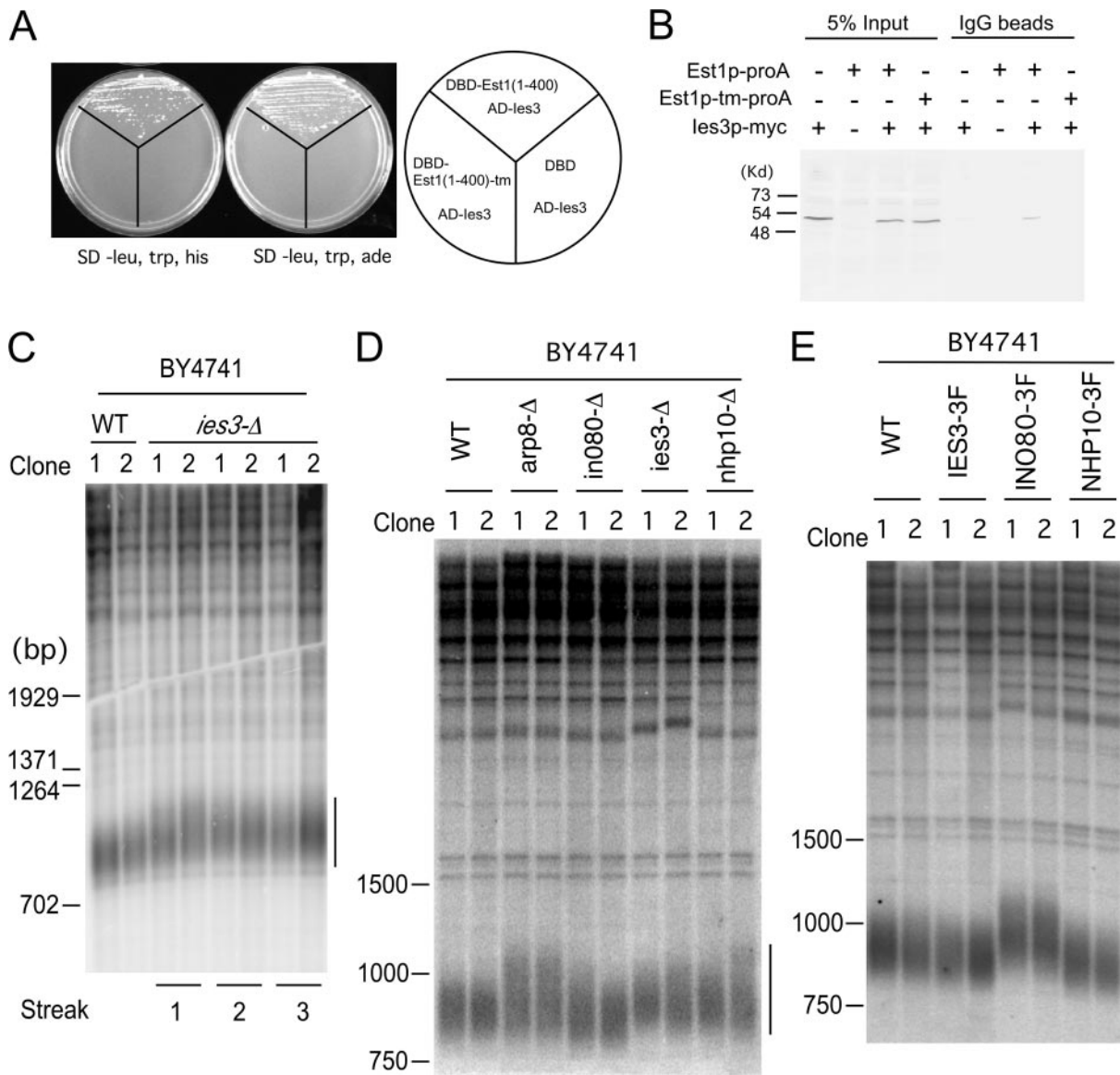


FIG. 1. Interaction between the TPR domain of Est1p and Ies3p and the effect of deleting INO80 complex subunits on telomere lengths. (A) Expression plasmids containing DBD-EST1 and AD-IES3 fusions were introduced into PJ69-4A, and the resulting strain was monitored for reporter gene expression (*ADE2* and *HIS3*) as judged by growth on selective medium. The plates are shown on the left, and the identities of the expression plasmids in the strains are shown on the right. Est1(1-400)-tm carries three point mutations at conserved positions in the Est1p TPR domain (198^{G→A}, 236^{P→A}, and 278^{N→A}). (B) *EST1* and *IES3* were tagged separately with the proA tag (proA) and the myc tag (myc) and placed on centromeric plasmids. Combinations of the plasmids were introduced into W303a followed by extract preparation and immunoglobulin G-Sepharose binding. Both the input and bead samples were analyzed by Western blotting using anti-myc antibodies. The identities of the tagged proteins expressed in the strains are indicated at the top. Est1p-tm-proA carries three point mutations at conserved positions in the TPR domain (198^{G→A}, 236^{P→A}, and 278^{N→A}). (C and D) Several strains that are missing individual subunits of the INO80 complex were obtained by gene disruption, request, or purchase (see Materials and Methods). Chromosomal DNAs from the strains were digested by PstI and assayed for the distribution of telomere restriction fragments by Southern analysis. In the case of *ies3-Δ* clones, the DNAs were isolated from cells that were passaged by serial restreaking. The position of Y' telomeres is marked by a vertical bar to the right. (E) Several strains in which an INO80 subunit is tagged with FLAG₃ were analyzed for telomere lengths as described for panels C and D. Abbreviations: AD, activation domain; DBD, DNA binding domain; WT, wild type; IgG, immunoglobulin G. Numbers at left of panels D and E are lengths in base pairs.

locus in the IP sample was calculated to be 1/40 of the input and the control locus in the IP sample was calculated to be 1/80 of the input, then the enrichment is considered twofold. This method is primarily used to correct for slight deviations from linearity of the PCRs. For ChIP analysis of myc₉-tagged Rsc3p, the washing and elution conditions were those described by Fisher et al. (18) and the primers for amplification of the HTA1-HTB1 intergenic region were 5'-TGCCACTACTAAGGCCAATTCTCTT-3' and 5'-ATACACTCCATTCCAATAGCTTCGC-3'.

RESULTS

Ies3p is an Est1p-interacting protein. To identify potential interacting partners for the conserved TPR domain of yeast Est1p, we used this domain as bait in a two-hybrid screen. Out of ~10⁶ potential preys, we uncovered one that, when reintro-

duced together with the bait plasmid into a reporter strain, activated the expression of both the *ADE2* and *HIS3* reporter genes (Fig. 1A). This prey contains a portion of the *IES3* (*INO80* subunit 3) gene, which encodes a subunit of the *INO80* chromatin remodeling complex. A mutant allele of Est1 TPR (Est1-tm) bearing three alanine substitutions at conserved positions (198^{G→A}, 236^{P→A}, and 278^{N→A}) was generated and found to be defective in the two-hybrid assay, arguing that the interaction is dependent on an intact TPR structure (Fig. 1A). To further assess physical interaction between Est1p and Ies3p, we tagged them with a protein A (proA) tag and a myc₉ (myc) tag, respectively, and examined their ability to coprecipitate from extracts of cells expressing both protein variants. As shown in Fig. 1B, immunoglobulin G-Sepharose precipitated a small amount of Ies3p-myc in an Est1p-proA-dependent fashion, supporting the idea of a weak interaction between the two proteins.

We attempted to address the functional significance of the Est1p-Ies3p interaction by examining the effect of *EST1* mutations in the TPR domain. However, we found that the Est1-tm allele and several other mutants in the TPR domain all exhibited reduced binding to TLC1 RNA, which in turn caused telomere shortening (data not shown). This result is consistent with recent analyses that support a novel role for the TPR domain of Est1p in RNA binding (17; N. F. Lue et al., unpublished data). An *est1* mutant that is specifically defective in Ies3p binding will be necessary to address the functional significance of the Est1p-Ies3p interaction.

Mutations in some components of the *INO80* complex induce telomere elongation. We then explored possible roles of *IES3* and associated factors in telomere regulation. As noted before, Ies3p is a component of the *INO80* chromatin remodeling complex, which contains more than 10 subunits, including Ino80p (the ATPase subunit); Rvb1p and Rvb2p (helicase-like); Arp4p, Arp5p, and Arp8p (actin related); Nhp10p (HMG-like); and Ies1p through Ies6p (35, 46). The specific functions of most of the subunits have not been elucidated. However, Arp8p is known to be required for the ATPase activity of the complex. To assess the roles of Ies3p and other subunits of the *INO80* complex at telomeres, we analyzed the lengths of telomere restriction fragments in mutants that are missing individual components of the complex. First, *ies3-Δ* mutants were constructed using two different laboratory strains, W303a and BY4741, and the lengths of telomeres in the mutants were analyzed over several passages. Interestingly, loss of Ies3p in each strain background resulted in moderate telomere elongation (Fig. 1C; see also Fig. S1A in the supplemental material). Specifically, the Y' telomeres reached a new equilibrium length distribution that is on average ~100 bp longer than the wild-type distribution after just 25 to 50 generations (one to two streaks). Thus, Ies3p is apparently a negative regulator of telomere lengths. We then assessed the telomeres of mutant strains that are missing other components of the *INO80* complex. Like Ies3p, three other components of the complex, including Arp8p, Nhp10p, and Ies6p, appear to be negative regulators of telomere lengths because deletion of the corresponding genes leads to telomere elongation (Fig. 1D; see also Fig. S1B in the supplemental material). Analysis of the role of the catalytic *INO80* subunit revealed complex effects of this protein: while the deletion strain (*ino80-Δ*) manifested no

change in telomere length (Fig. 1D), a strain in which the *INO80* gene is modified at the 3' end with a FLAG₃ tag (*INO80-3F*) manifested significant telomere elongation (Fig. 1E). Because the tagged strain exhibited normal growth and response to DNA damage (data not shown), the *INO80-3F* allele appears to retain much of the function of the native gene. Thus, our results suggest that some alteration of *INO80* function can induce telomere elongation whereas complete loss of function causes no net changes. A possible explanation for these apparently disparate phenotypes is presented in the Discussion. Altogether, our data indicate that multiple subunits of the *INO80* complex are negative regulators of telomere lengths.

Alterations in telomere structure can sometimes induce changes in the level of G-strand overhangs as well as in TPE. The G-strand overhangs were found to be unchanged in *ies3-Δ*, *ies6-Δ*, *nhp10-Δ*, and *ino80-Δ* strains by in-gel hybridization analysis of telomeric DNA (data not shown). However, TPE in the *ies3-Δ* strain was consistently reduced relative to the wild-type control (see Fig. S2 in the supplemental material; also data not shown). In addition, we observed loss of TPE in two clones of the *arp8-Δ* mutant (see Fig. S2 in the supplemental material; also data not shown). However, this effect appears to be stochastic as several other *arp8-Δ* clones showed no alteration in TPE. Thus, subunits of the *INO80* complex can influence the structure as well as the length of telomeres in yeast.

Loss of Ies3p impedes survivor formation and alters the structure of telomeres in survivors. In yeast mutants that are missing components of the telomerase complex (e.g., Est1p, Est2p, Est3p, and Tlc1), telomeres suffer progressive attrition, which results in severe growth defects (31). However, if the strain is continuously subcultured (by serial restreaking on plates or serial dilution in liquid cultures), then rare populations of "survivors" invariably arise (30, 55). These survivors exhibit growth rates that resemble those of a wild-type strain and are believed to utilize recombination-based mechanisms for telomere maintenance. Loss of several recombination proteins, including Rad52p, Rad51p, and Rad50p, has been shown to impair survivor formation (23, 29). The *INO80* complex has been implicated in promoting the kinetics of recombinational repair of DSBs by modulating chromatin structure near the breaks, raising the possibility that it might also regulate recombination at telomeres (35, 58). To determine if Ies3p has a role in this process, we created isogenic *est1-Δ* and *est1-Δ ies3-Δ* mutants by mating and sporulation and compared their growth properties in liquid cultures over a period of 12 days. On each day, the clones were diluted to 2×10^5 cells/ml and allowed to grow for 20 h, followed by measurement of cell density (21). As shown in Fig. 2A, an *est1-Δ* clone suffered severe growth retardation on days 3 and 4 during passage but regained normal growth on day 5 as anticipated. The isogenic *est1-Δ ies3-Δ* clone also experienced severe growth impairment but failed to recover until day 7. Four isogenic pairs of *est1-Δ* and *est1-Δ ies3-Δ* clones were examined, and the difference in the recovery kinetics of the single and double mutants was reproducible and statistically significant (Fig. 2B). On average, the *est1-Δ* clones exhibited growth recovery after 3 days, whereas the *est1-Δ ies3-Δ* clones exhibited recovery after 6 days.

Previous analysis of telomere structure in yeast survivor

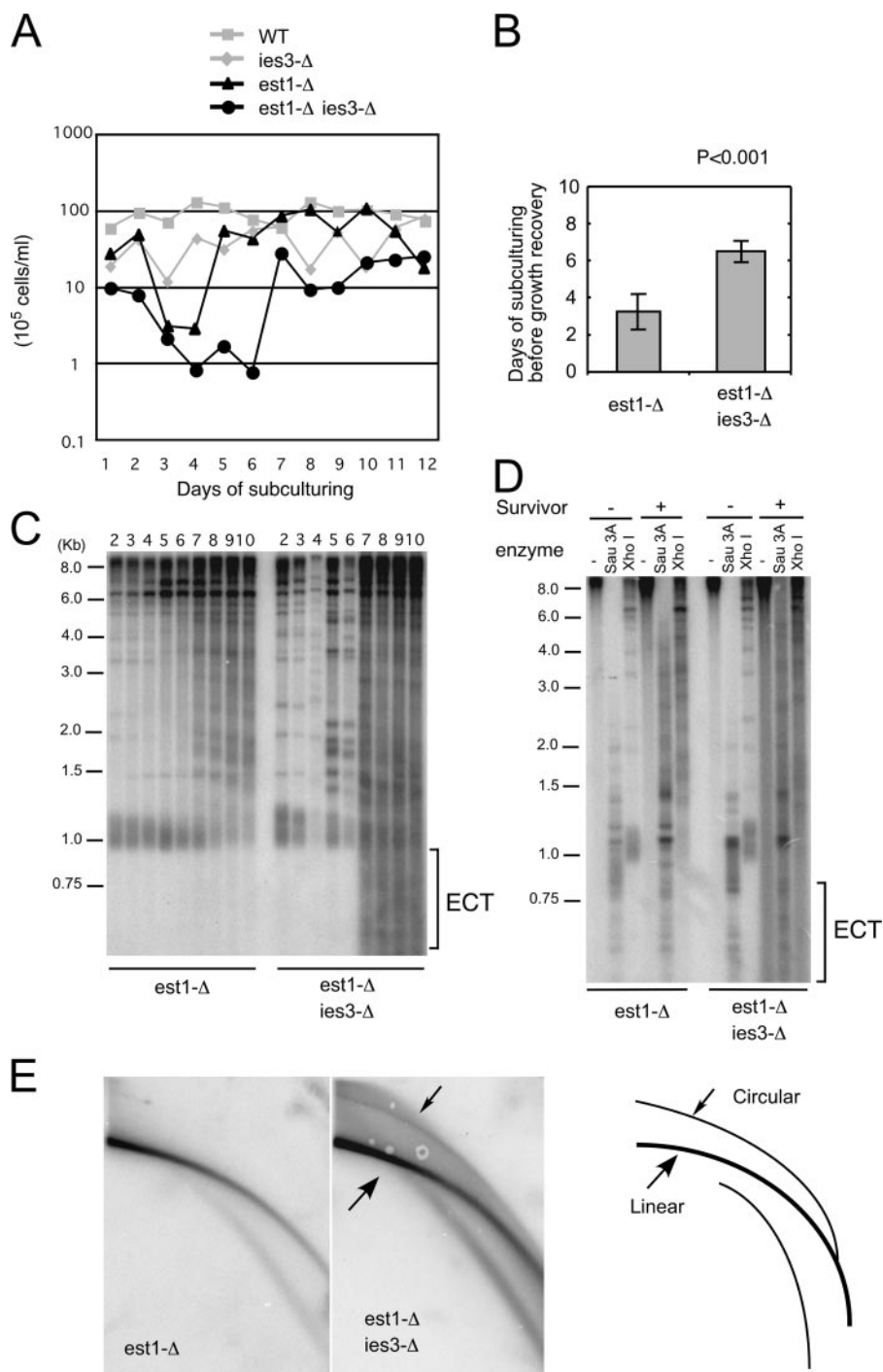


FIG. 2. Effects of *IES3* deletion on telomerase-deficient survivors. (A) Isogenic wild-type (WT), *ies3*-Δ, *est1*-Δ, and double mutant clones were obtained by sporulation and tetrad dissection. The clones were passaged in liquid cultures (by dilution to 2×10^5 cells/ml on each day followed by 20 h of growth) over a period of 12 days. The cell density on each day (prior to dilution) was plotted. (B) The numbers of days after which the *est1*-Δ and *ies3*-Δ *est1*-Δ mutants experienced recovery were determined for four clones each. The averages and standard deviations were plotted. (C) Chromosomal DNAs were isolated from successive days of the liquid culture derived from the experiment shown in panel A and assayed for the distribution of telomere restriction fragments after XhoI digestion. (D) Chromosomal DNAs were isolated from the *est1*-Δ and *est1*-Δ *ies3*-Δ cultures on days 2 and 10, treated with restriction enzymes as indicated at the top, and assayed for the length distribution of telomere-containing fragments. Numbers at left are lengths in kilobases. (E) Chromosomal DNAs were isolated from the *est1*-Δ and *est1*-Δ *ies3*-Δ cultures on day 10 and subjected to two-dimensional gel electrophoresis followed by Southern analysis using labeled poly(dG-dT) · poly(dA-dC) as the probe.

clones revealed two types of cells: type I cells, which contain short terminal $G_{1-3}T$ tracts but increased numbers of subtelomeric Y' repeats, and type II cells, which contain long and heterogeneous terminal $G_{1-3}T$ tracts (30, 54). Because of higher growth rates, type II survivors tend to be predominant in serially passaged liquid cultures. This was found to be the case for a pair of *est1-Δ* and *est1-Δ ies3-Δ* survivor clones, which exhibited long and heterogeneous terminal restriction fragments (Fig. 2C). Interestingly, the survivors of the double mutant manifested two unusual features: the overall levels of the telomeric repeat content were substantially increased relative to the *est1-Δ* single mutant, and there was an emergence of low-molecular-weight telomeric DNAs (~200 to 1,000 bp long; compare lanes 7 to 10 of left and right panels in Fig. 2C). These telomeric DNAs must be extrachromosomal (henceforth referred to as ECT for extrachromosomal telomeric DNA) because they were detected even in the absence of restriction enzyme digestion (Fig. 2D). Indeed, two-dimensional gel electrophoretic analysis of the DNA samples revealed high levels of circular telomeric DNA in the double mutant survivor but not the single mutant (Fig. 2E; see also Fig. S3 in the supplemental material; data not shown).

Several groups have recently described unusual telomeric DNA structures in yeast mutants missing Cdc13p, a single-stranded end-binding protein. They report that in such mutants, there is often a dramatic increase in the levels of unpaired G-strand. We therefore analyzed the levels of G-strand overhangs in the *est1-Δ ies3-Δ* survivor clones (see Fig. S4 in the supplemental material). Taken together, our findings demonstrate that loss of Ies3p not only impacts on the kinetics of survivor formation but also can alter the structure of telomeric DNA in the survivors.

To test the generality of the effect of *IES3* deletion on the structure of telomeres in the survivors, we generated and analyzed multiple survivor clones of different genotypes by continuous liquid subculturing. The tested strains included the *est1-Δ* and *est2-Δ* single mutants and the *est1-Δ ies3-Δ* and *est2-Δ ies3-Δ* double mutants. Remarkably, we found that while none of the 15 single mutant survivors manifested evidence of ECT, 5 out of 10 *est1-Δ ies3-Δ* survivors and four out of seven *est2-Δ ies3-Δ* survivors showed evidence of such DNA (data not shown). Thus, deletion of *IES3* does not invariably lead to the formation of ECT but appears to stimulate this process.

As described above, the survivors that we analyzed from serially passaged liquid cultures were all type I survivors, which are expected to be predominant when telomerase mutants are passaged in liquid cultures. However, we were able to identify multiple clones of type I survivors by passaging both the *est1-Δ* and *est1-Δ ies3-Δ* mutants on solid medium (data not shown). Thus, loss of Ies3p does not abrogate the formation of type I survivors.

The combined loss of Ies3p and telomerase leads to elevated levels of T-T fusion. DNA DSBs can be repaired either by homologous recombination or by nonhomologous end joining (59). Like DSBs, very short telomeres and telomeres with altered structures have been shown to undergo not only recombinational "repair" (which gives rise to survivors), but also nonhomologous end joining (which gives rise to T-T fusion). Because loss of Ies3p appears to impair recombinational "re-

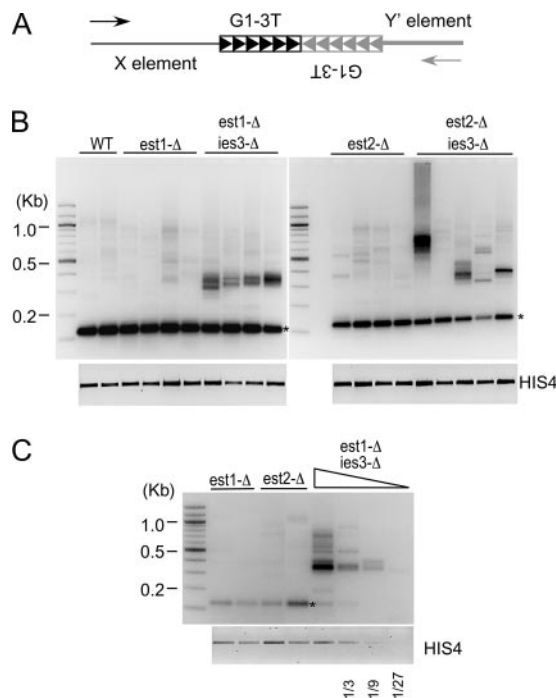


FIG. 3. Identification of T-T fusions. (A) A diagrammatic illustration of the PCR protocol used to detect T-T fusions. (B) Chromosomal DNAs from wild type (WT) and mutants were isolated, digested with *Sau3AI*, and tested for the presence of T-T fusions as described in Materials and Methods. As a control for the quality of chromosomal DNA preparations, a *HIS4* fragment was separately amplified using these preparations, and the results are shown at the bottom. The mutants were estimated to have undergone ~50 to 75 cell divisions prior to the isolation of DNA. In almost all fusion assays, an abundant ~120-bp fragment was observed (denoted by the star). Cloning and sequencing of the fragment indicate that it was due to amplification of a small segment of chromosome XVI which is flanked by sequences that are partially complementary to the X and Y' primers (data not shown). (C) The fusion assays were performed using two *est1-Δ* samples, two *est2-Δ* samples, and one *est1-Δ ies3-Δ* sample that had been subjected to serial dilutions. The identities of the mutants are shown at the top, and the dilution factors are shown at the bottom.

pair" of aberrant telomeres, we wondered if the protein might also directly or indirectly affect T-T fusion. To address this possibility, we analyzed the presence of fusions in mutants that are missing Ies3p, Est1p, Est2p, or combinations thereof. The fusions were detected by PCR using primer pairs that anneal to the Y' and X subtelomeric sequences and that have the appropriate polarities (Fig. 3A) (33). To control for the quality of the DNA preparations and the efficiency of PCR, we separately amplified a *HIS4* fragment using the same chromosomal DNA preparations (bottom panels in Fig. 3B and 3C). We found that multiple isolates of the wild-type, *ies3-Δ*, and *ino80-Δ* strains did not exhibit appreciable levels of T-T fusion, indicating that loss of INO80 subunits alone is not sufficient to promote this aberrant repair (Fig. 3B and data not shown). Consistent with earlier studies, loss of either Est1p or Est2p also resulted in very low levels of fusion (Fig. 3B). In contrast, much higher levels of fusion were detected in the *est1-Δ ies3-Δ* and *est2-Δ ies3-Δ* double mutants. Analysis of the PCR signals generated by serial dilutions of the samples indicates that the fusion levels are increased by ~10- to 30-fold in the double

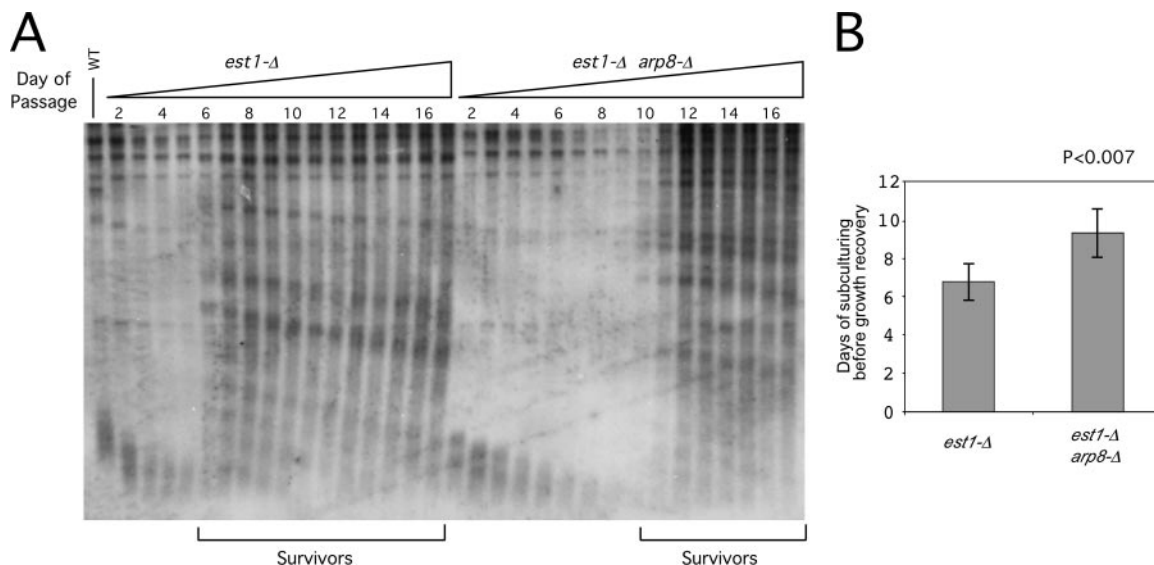


FIG. 4. The *est1-Δ arp8-Δ* double mutant exhibits a delay in the emergence of survivors. (A) Isogenic *est1-Δ* mutant and *arp8-Δ est1-Δ* double mutant clones were obtained by gene replacement. The telomeres in the clones were initially maintained by plasmid copies of *EST1*. The clones were selected for loss of the plasmid on 5-FOA-containing plates and then passaged in liquid cultures (by dilution to 2×10^5 cells/ml on each day followed by 20 h of growth) over a period of 18 days. Chromosomal DNAs were isolated and subjected to telomere Southern analysis. Telomere dynamics in a representative pair of mutants are shown. WT, wild type. (B) Four *est1-Δ* and seven *est1-Δ arp8-Δ* clones were monitored for growth by continual passages in liquid culture. The duration before growth recovery was determined, and the average and standard deviation for each genotype were plotted. The *P* value for the difference is shown at the top.

mutants in comparison with either of the single mutants (Fig. 3C). The putative fusion PCR products were recovered by cloning and subjected to sequence analysis. This analysis revealed fusions of telomeres that have invariably experienced significant shortening such that less than 60 bp of repeat tracts was retained at the junctions (see Fig. S5 in the supplemental material). Elevated fusion levels can also be detected in the double mutants by using a different PCR protocol (40) (data not shown). We conclude that loss of telomere repeat tracts in combination with *IES3* deletion can induce high levels of chromosomal end-to-end fusions.

Loss of Arp8p, a protein required for the chromatin remodeling activity of INO80 complex, delayed survivor formation. The multiple consequences of *IES3* deletion in telomerase-negative cells may be due to the contribution of *IES3* to chromatin remodeling or some other functions of the subunit at telomeres. To begin to distinguish between these alternatives, we investigated the phenotypes of the *est1-Δ arp8-Δ* mutant; because Arp8p is required for the chromatin remodeling activity, any phenotypic similarity between the *est1-Δ arp8-Δ* mutant and the *est1-Δ ies3-Δ* mutant would support chromatin remodeling as the underlying mechanism. Interestingly, like the *est1-Δ ies3-Δ* mutant, multiple independent *est1-Δ arp8-Δ* clones exhibited a substantial delay in the kinetics of survivor formation (Fig. 4A and B). In this experiment, whereas the *est1-Δ* survivors typically emerged after 6 to 7 days of subculturing, the *est1-Δ arp8-Δ* survivors emerged after 9 to 10 days. This difference in timing was reproducible and statistically significant (Fig. 4B). We note that the duration prior to survivor emergence for the *est1-Δ* single mutant is longer in this experiment than that in the experiment shown in Fig. 2. This is likely due to the difference in strain background and method of

strain derivation. In any case, our result supports the notion that subunits of the INO80 complex facilitate survivor formation by remodeling chromatin structure. In contrast, preliminary analysis did not reveal high levels of ECT or high levels of T-T fusions in multiple *est1-Δ arp8-Δ* clones (data not shown), suggesting that the ability of *IES3* to suppress these structures may be mediated by a mechanism that is distinct from chromatin remodeling.

Subunits of the INO80 complex associate preferentially with telomeres. Like other ATP-dependent chromatin remodeling complexes, the INO80 complex is known to regulate the expression of a large number of genes (34). Therefore, the phenotypic consequences of INO80 subunit mutations could conceivably be due to changes in the expression levels of telomere- and telomerase-related genes. To determine if the INO80 complex functions directly at telomeres, we investigated the telomere association of INO80 complex components in vivo using ChIP. For this purpose, we generated strains containing FLAG₃-tagged Ies3p, Ino80p, or Nhp10p. As noted above, tagging of INO80 caused telomere elongation but did not impair cell growth or DNA damage sensitivity. The same modification of *IES3* and *NHP10* had no effect on telomere lengths. The association of tagged proteins with telomeric DNAs was assessed by PCR following cross-linking and immunoprecipitation. Three separate telomeric loci (the X element on the right arm of chromosome VI [Tel-VI], the X element on the left arm of chromosome XV [Tel-XV], and a subtelomeric region on the right arm of chromosome VI [Subtel-VI]) were tested. A nontelomeric control locus (an intergenic sequence on the left arm of chromosome V [Intergene-V]) was also analyzed. As shown in Fig. 5A, all three FLAG₃-tagged INO80 subunits specifically immunoprecipitated higher levels of

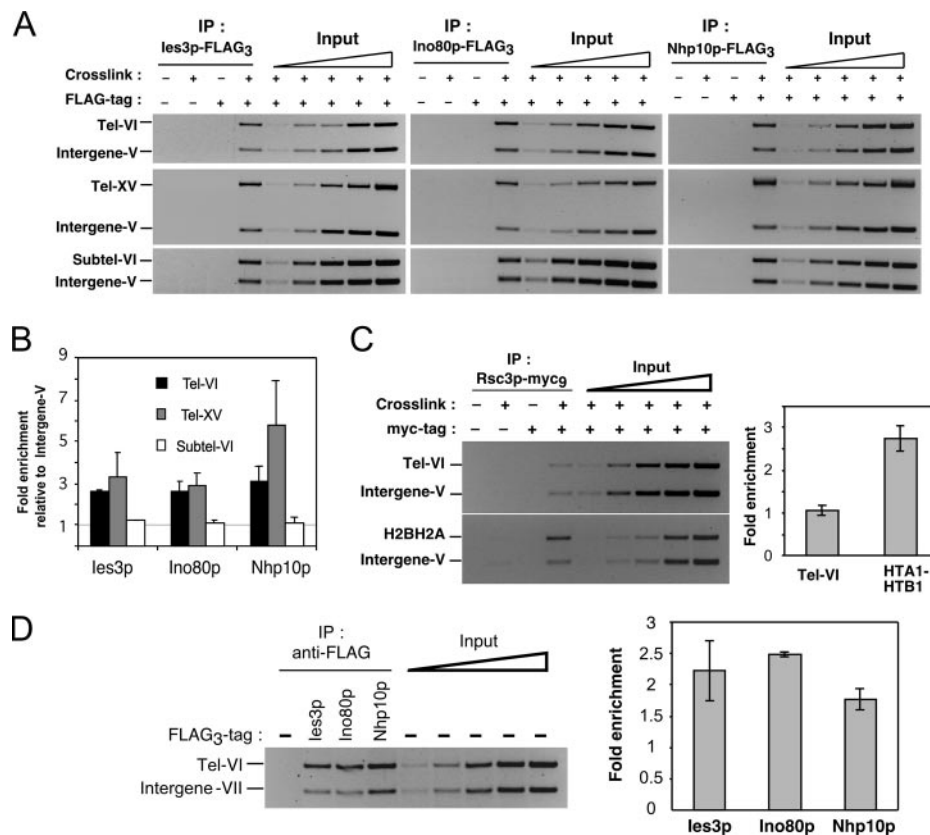


FIG. 5. Subunits of the INO80 complex are preferentially enriched at telomeres. (A) ChIP assays were performed on strains containing FLAG₃-tagged *Ies3p*, *Ino80p*, or *Nhp10p* using antibodies directed against the FLAG peptide. BY4741 and FLAG₃-tagged strains not subjected to cross-linking were used as negative controls. In each experiment, twofold serial dilutions of the input DNA and the immunoprecipitated DNAs were analyzed by PCR using a primer pair specific for the telomeric regions (Tel-VI, Tel-XV, or Subtel-VI) and one specific for a nontelomeric locus (Intergene-V). (B) PCR results from at least three replicate experiments like those shown in panel A were quantified to obtain the magnitude of enrichment (Materials and Methods). The relative enrichment for the telomeric loci over Intergene-V was plotted. Error bars represent the standard errors calculated from at least three independent experiments. (C) ChIP assays were performed using a strain that contains myc₉-tagged *Rsc3p*. Association of *Rsc3p* with Tel-VI or HTA1-HTB1 (a known binding site for the RSC complex) relative to Intergene-V was analyzed by at least three independent experiments. Representative assays are shown on the left, and the quantitative results are shown on the right. (D) ChIP assays were performed using strains containing FLAG₃-tagged *Ino80p*, *Ies3p*, or *Nhp10p*. Association of the tagged proteins with telomere VI (Tel-VI) relative to a chromosome VII locus (Intergene-VII) was analyzed by multiplex PCR. A set of representative assays is shown at the left. The average enrichment for each protein at Tel-VI was determined from at least three independent experiments, and the results are plotted at the right.

Tel-VI and Tel-XV in comparison with Intergene-V. No PCR signal was detected in anti-FLAG antibody immunoprecipitates from an untagged strain or FLAG₃-tagged strains without cross-linking, indicating that this enrichment is specific and cross-link dependent. The average magnitude of enrichment for Tel-VI relative to Intergene-V is 2.6, 2.6, and 3.1 for *Ies3p*, *Ino80p*, and *Nhp10p*, respectively (Fig. 5B). Tel-XV was also enriched by about 3.3-, 2.9-, and 5.8-fold over Intergene-V for *Ies3p*, *Ino80p*, and *Nhp10p*, respectively. In contrast, Subtel-VI showed no significant enrichment in this analysis for all of the tagged strains. Enrichment of *Ino80p*, *Ies3p*, and *Nhp10p* at telomeres was also observed when a different control locus on chromosome VII was used in the analysis (Fig. 5D). As a further control, we investigated the chromosomal localization of *Rsc3p*, a subunit of another ATP-dependent chromatin remodeling complex named RSC. ChIP analysis of a strain with a myc₉-tagged *Rsc3p* did not reveal preferential localization of this protein to telomeres (Fig. 5C). Taken all

together, our data indicate that at least three INO80 complex subunits are preferentially associated with DNA within or near the terminal repeats of telomeres *in vivo*, consistent with direct action of this complex at telomeres. While tagging apparently altered the function of INO80 with respect to telomere length regulation, the similar ChIP results for tagged INO80, IES3, and NHP10 support their concurrent localization to telomeres.

The interaction between *Est1p* and *Ies3p* raises the possibility that *Est1p* may be required for the recruitment of the INO80 complex to telomeres. However, the synthetic phenotypes of the *est1-Δ ies3-Δ* double mutant with respect to survivor formation and T-T fusions suggest that the INO80 complex can affect telomere structure and function even in the absence of *Est1p*. To examine this issue directly, we analyzed the localization of FLAG₃-tagged *Ino80p* at telomeres in an *est1-Δ* strain. As shown in Fig. 6, association of *Ino80p* with Tel-VI was retained in several *est1-Δ* transformants that have undergone ~30 cell divisions, indicating that *Est1p* is not essential

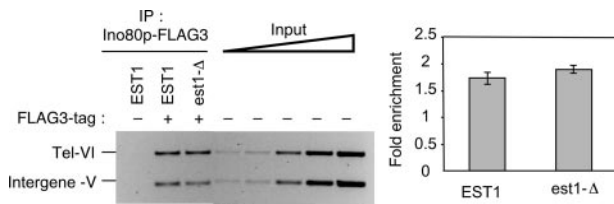


FIG. 6. Est1p is not essential for telomere localization of INO80. *est1-Δ* strains in the BY4741 background that carry the FLAG₃-tagged Ino80p were generated by homologous recombination using a *URA3*-based disruption cassette. A single transformant colony was inoculated into 50 ml of medium, and the culture was grown to $\sim 2 \times 10^7$ cells/ml (~ 30 generations). Disruption of *EST1* was confirmed by PCR and telomere Southern analysis, and the cells were subjected to ChIP as described in Materials and Methods. Representative assays are shown at the left, and the quantitative results from two independent experiments are plotted at the right. Note that the ChIP experiments for *EST1* cells were performed concurrently with those for the *est1-Δ* cells and differ from the ones presented in Fig. 5A and B; hence, the enrichment value for Ino80p at Tel-VI is slightly different.

for the recruitment of INO80 complex to telomeres. This result is consistent with the synthetic phenotypes of the *est1-Δ ies3-Δ* double mutant. Because the association of Est1p with telomeres is cell cycle dependent and may be restricted to short telomeres, more studies are necessary to determine if Est1p can have a stimulatory role in INO80 recruitment under some conditions (18, 52).

DISCUSSION

We show that multiple subunits of an ATP-dependent chromatin remodeling complex regulate telomere structure and functions. Loss of some subunits of the INO80 complex induced telomere elongation in telomerase-positive yeasts. Loss of an INO80 subunit was also accompanied by a delay in the emergence of telomerase-deficient survivors and increased levels of ECT DNA and T-T fusions in the survivors. Thus, in addition to its emerging role in DSB repair, the complex may also serve multiple functions in telomere regulation. Preferential localization of multiple components of the complex to telomeres suggests that the complex can act directly at telomeres. However, whether and how each individual subunit of the complex contributes to telomere regulation are subjects that await further investigation.

In one respect, the phenotypic consequence of INO80 subunit mutations at telomeres is similar to that at DSBs: the mutants exhibit a reduced ability to deal with “damaged” DNAs, be they interstitial breaks or critically short telomeres. While some studies suggest that the INO80 complex works by removing nucleosomes at the DSB to facilitate the loading of recombination proteins, another study points to a role for INO80 in checkpoint adaptation (35, 39, 57, 58). Defects in either process could conceivably explain reduced survivor formation in mutants with the combined loss of telomerase and INO80 subunits. However, other phenotypes of INO80 subunit mutations suggest that these subunits could have additional functions. First, *IES3* appears to have a role in regulating the structure of telomeres in survivors (i.e., the level of ECT circles), after recombinational repair has taken place. Second, deletion of several INO80 subunits (including Arp8p, which is

required for the ATPase activity of the complex) can induce telomere elongation in telomerase-positive cells, suggesting that the complex also acts at normal telomeres. It should be noted that none of the INO80 subunits were identified by the two genome-wide screens aimed at discovering genes that influence telomere length (1, 19). However, such screens are known to yield significant numbers of false positives and negatives (15). In addition, the length changes that we observed for INO80 subunit mutants, though reproducible, are relatively modest and could have been dismissed by investigators looking for large changes. In fact, one of the screens noted the *nhp10-Δ* mutant as exhibiting questionable lengthening of telomeres (1).

Why does tagging of INO80 cause telomere elongation while deletion of the gene has no effect? One possibility is that the gene may influence telomere length both directly and indirectly, but in opposite ways. More specifically, like Ies3p, Ino80p may act directly and negatively to regulate telomere length. But Ino80p is also a transcription regulator and may indirectly but positively regulate telomere length by influencing the expression of telomere-related genes. Indeed, examination of previously published screening and microarray data identified at least 12 targets of INO80 that may contribute to this positive regulatory role of Ino80p (see Table S1 in the supplemental material). The disparate effect of tagging and deletion can then be explained by postulating that tagging preferentially affects the negative activity of Ino80p at telomeres.

A particularly intriguing observation that emerged from the current analysis concerns the striking features of telomeric DNAs in the *est1-Δ ies3-Δ* survivors, which are greatly amplified and heterogeneous and exist at high levels in both circular and single-stranded forms. While low levels of circular and single-stranded telomeres have been reported in typical survivors (26), the unique features of the *est1-Δ ies3-Δ* survivors have only two precedents. First, yeast strains that possess altered telomere repeats due to mutation in telomerase RNA can exhibit highly elongated, circular, and degraded telomeres (20, 27). Second, yeast mutants lacking Cdc13p manifest extreme amplification of telomere repeats, which exists in both extrachromosomal and single-stranded states (25, 42, 63). These results suggest that sequence-specific telomere binding proteins (especially Cdc13p) play a role in suppressing these highly aberrant structures. The phenotypes of the *est1-Δ ies3-Δ* survivors hint at a comparable role for *IES3*. It has been observed that a high percentage of human ALT cell lines, which lack telomerase and use recombination to maintain telomeres, have an appreciable amount of ECT circles (10, 62). Whether a human chromatin remodeling complex regulates the ALT pathway is an interesting question for future investigation.

The evolutionary conservation of the TPR domain of Est1p and multiple INO80 subunits raises the possibility that regulation of telomere structure and functions by a chromatin remodeling complex will turn out to be widespread in nature (2, 22). Indeed, a human complex that resembles the yeast INO80 complex in terms of subunit composition and biochemical activity was recently identified (22). Moreover, recent studies suggest that in addition to the INO80 complex, other chromatin remodeling complexes, including the SWI/SNF and RSC complexes, are also present at DSBs and facilitate different steps of the recombination repair process (11, 47). Interestingly, while an *rsf2-Δ* mutant has been reported to have short

telomeres, our preliminary analysis indicates that the RSC complex may not be preferentially associated with normal telomeres. Thus, the RSC complex may indirectly regulate telomere lengths, possibly by controlling the expression of telomere-related genes. It remains to be determined if this and other chromatin remodeling complexes have a role in regulating or repairing dysfunctional telomeres.

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