Saecharomyces Rrm3p, a member of Pif1 5′–3′ DNA helicase subfamily, helps replication forks traverse protein-DNA complexes, including the telomere. Here we have identified an Rrm3p interaction protein known to be Def1p. In def1 mutants, telomeres were ~200-bp shorter than that in wild-type cells. DEF1 is also required for the stable maintenance of mitochondrial DNA, and the telomere shortening phenotype seen in def1 cells is not a secondary consequence of the mitochondrial defect. A combination of DEF1 null mutation with deletion of EST2 or EST3 resulted in an accelerated senescence phenotype, suggesting that Def1p is not involved in the telomerase recruitment pathway. In the absence of telomerase, cells escape senescence by either amplifying Y′ regions or TG-telomeric repeats to generate type I or type II survivors, respectively. Only type I survivors were recovered from both def1Δ est2Δ and def1Δ est3Δ double mutant cells, further suggesting that the function of Def1p in telomere maintenance is specific. Our novel findings of the functions of Def1p in telomere and mitochondria suggested that Def1p plays multiple roles in yeast.

Telomeres are the physical ends of eukaryotic chromosomes that are composed of specific repeat DNA sequences and binding proteins (1). The telomeres mainly serve three functions. First, the telomere protects chromosome both from degradation and from fusion with other ends (2, 3). Second, in most eukaryotes, it facilitates the complete replication of chromosomes through telomerase, a special reverse transcriptase using Tlc1 as the RNA template, or homologous recombination, which is RAD52-dependent (1, 4, 5). However, Drosophila has an unusual telomere elongation mechanism. Long retrotransposons (HeT-A and TART) transpose to the ends of chromosomes instead of short repeats, which are synthesized by telomerase (6).

Third, the telomere forms a heterochromatic regions that represses the subtelomeric genes, at least in some species (7). In Saecharomyces cerevisiae, for example, each end of every chromosome bears ~300 ± 75 bp of C1-3A/TG1–3 telomeric DNA. In addition to the TG1–3 tracts, yeast chromosomes have telomere associated repeats called X and Y′ (8).

In S. cerevisiae, the telomere integrity is maintained through both telomere elongation/shortening and telomere end capping activities (9, 10). Many factors that function in telomeres contribute to both telomere replication and capping processes. Telomerase, a specialized reverse transcriptase that is comprised of several subunits including Est1p, Est2p (catalytic subunit), Est3p, and Tlc1 (the RNA template), is responsible for the elongation of TG1–3 tracts (11–13). Disrupting any of them will cause progressive telomere shortening and eventually cellular senescence, although only the catalytic subunit Est2p and the RNA subunit Tlc1 are required for the in vivo telomerase activity (14, 15). Besides its function on telomeric DNA elongation, the telomerase also contributes to telomere capping (16). Another key regulator of telomere homeostasis is the Cdc13-Stn1-Ten1 complex, which is involved in both telomere length regulation and telomere end protection. The cdc13-1, sta1-13 and ten1-31 mutants showed telomere length change and accumulation of single-stranded G-rich overhang of telomeric regions when incubated at a 36 °C-restrictive temperature (17–19). The biochemical characterization of Cdc13p showed that it binds telomeric single-stranded G-rich DNA with extremely high affinity (20, 21). The in vivo cross-linking experiments demonstrated that Cdc13p binds telomeres (22). These findings suggest that Cdc13p is bound to the single-stranded G-rich overhang in vivo. The Rap1 protein, which binds with high affinity to specific sequences within the telomeric TG1–3 tracts (23), is a major positive regulator. Mutation of Rap1p, as well as its associate proteins Ril1p and Ril2p, leads to telomere elongation (24, 25). The Tel1p and Mec1p, two ATM-like protein kinases are checkpoint regulators (26–28). Disrupting one of them results in stable shortened telomeres, double deletion causes the progressive shortening of telomeres and cell senescence (28), and the double mutating of mec1tel1 ultimately results in gross chromosomal rearrangements. The yKu70/80 heterodimer not only positively regulates telomere length, but also plays a protective role on telomeres. yKu mutant strains display short but stable telomeres (29, 30), and the single-stranded telomeric G-rich strand, usually restricted to S phase in wild-type cells, is present in yku cells throughout the cell cycle (31).

Recently, we found that the Pif1 subfamily helicases, including Pif1p and Rrm3p in S. cerevisiae and Phf1p in Schizosaccharomyces pombe, all affect telomeres (32–35). The Pif1p, a 5′–3′ DNA helicase, is the prototype of the helicase subfamily. The deletion of the PIF1 gene causes mitochondrial defect and telomere shortening (36, 37), and the telomere lengthening is TLC1 (the gene encodes the RNA subunit of telomerase)-dependent (32, 33). Besides its telomere function, the Pif1p is also required for the stable maintenance of mitochondrial DNA (36). Rrm3p is another member of Pif1 helicase subfamily in S. cerevisiae. Loss of Rrm3p resulted in very modest telomere lengthening, which is likely due to the replication defect in the
Def1p Has Function on Telomeres

Telomere Blot—Genomic DNA prepared from saturated cultures was digested by XhoI, separated on a 0.9% gel, transferred to Hybond-N+ membrane (Amersham Biosciences), and hybridized to a poly(GT) telomere-specific probe as described previously (35).

Growth Ability Assay—We inactivated the DEF1 gene in both estΔ and estΔ mutant cells. For the serial dilution plate assay, the wild-type, est2Δ, est3Δ, def1Δ, def1Δ est2Δ, and def1Δ est3Δ colonies were resuspended in 200 μl of water; 3 μl of each 10-fold serial dilution were plated on the YPDA plate and incubated at 30 °C for 2 days. At the same time when we were performing the serial dilution plate assay, cell viability in culture experiment was performed according the standard methods described before (40, 41). Each colony was inoculated into 5 ml of YPDA liquid culture and grown to saturation (106 cells/ml) at 30 °C. Each day was diluted to a concentration of 5 × 105 cells/ml in fresh YPDA medium, and the cell numbers were determined 24 h later by hemocytometer. This cycle was continued for 15–16 days and was repeated three times. At each dilution point, cells were examined for possible contamination and stored for telomere Southern blot. And the single-colony streak assay was performed according to the standard protocol described by Greider and co-workers (42).

Recombinant Protein Purification, Gel Shift Assay, and Gel Filtration—The DNA fragments, which encode for the truncated Def1p-N (aa 1–207) and Def1p-M (aa 350–586), were PCR-amplified from YPH499 genomic DNA and inserted at the EcoRI-XhoI site of pGEX-4T-1 vector. The GST fusion proteins were overexpressed and purified according to the manufacturer’s instructions. The purified recombinant proteins were analyzed with Coomassie Blue staining, and aliquoted for gel shift assay.

The Gel Shift Assay—Single-stranded DNA oligonucleotides were synthesized and two complementary oligonucleotides were annealed to form a double-stranded DNA, and the substrates were end-labeled with T4 polynucleotide kinase according to the manufacturer’s instructions. The binding reaction mixture (20-μl volume) contained 25 mM Tris (pH 7.9), 150 mM NaCl, 0.05 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1 μg of sperm single-stranded DNA, 1 μg of poly(dC-dI) and the indicated amounts of proteins. Reactions were incubated at 37 °C for 15 min, loaded onto a 5% nondenaturing polyacrylamide gel, and run in a 0.5× TBE buffer. The gel was dried on Whatman paper and imaged with a Typhoon PhosphorImager (Molecular Dynamics).

Gel Filtration—500 μg of purified Deflp-G (aa 381–480) in 100 μl of PBS was loaded on an analytical Superdex 75 column (Amersham Biosciences), which was calibrated according to the vendor’s protocol with protein standard markers including bovine serum albumin (67 kDa), ovalbumin (43 kDa), Chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). The column was developed with PBS at 0.3 ml min−1, and 1-ml fractions were collected. The elution profile was analyzed by Western blot.

RESULTS

Identification of Deflp—To study how telomere replication by conventional replication machinery and telomere elongation by telomerase are connected, an affinity purification of the proteins that interact with Rrm3p was carried out because Rrm3p promotes the replication fork progression through both telomeric and subtelomeric DNA (34). The RRM3 gene was tagged with 13myc epitope, and the yeast whole-cell extract was derived from a strain expressing 13myc-tagged Rrm3p and the yeast whole-cell extract was digested by XhoI, separated on a 0.9% gel, transferred to Hybond-N+ membrane (Amersham Biosciences), and hybridized to a poly(GT) telomere-specific probe as described previously (35).

Expression Yeasts, Media, and Plasmids—Unless otherwise noted, all S. cerevisiae deletion strains were of YPH499 and YPH500 background (39). Myc-tagged Rrm3p is of VPS16 background (32). The rrm3 allele was a deletion of the RRM3 open reading frame (by HIS3 in pRS303) made by using inverse PCR, the same method was used for the deletion of the open reading frames of DST1 (by HIS3 in pRS303), RAD26 (by LEU2 in pRS305), DEF1 (by TRP1 in pRS304), SNA3 (by HIS3 in pRS303), EST3 (by HIS3 in pRS303), and EST2 (by HIS3 in pRS303). Unless otherwise stated, all strains were grown at 30 °C and on YPDA medium (adeneine-supplemented TYP-10 G/l yeast extract, 20 G/l peptone, 2% dextrose). The knockout strains were analyzed with Western blot.

In this paper, we performed an affinity chromatography experiment by using 13myc-tagged Rrm3p as bait to isolate proteins that interact with Rrm3p. One of the proteins that interacts with Rrm3p is Deflp, which was reported to be required for degradation of RNA polymerase II in the DNA damage response (38). Here, we mainly show the novel functions of Deflp in telomeres. Our genetic and biochemical data suggest that Deflp is involved in telomere length regulation.

MATERIALS AND METHODS

A2 liter of either wild-type (YPH499) or mutant strains (data not shown). One of the proteins was called RR M3-13myc sample, but not in the RRM3-13myc sample, and the yeast whole-cell extract was derived from a strain expressing 13myc-tagged Rrm3p and the yeast whole-cell extract was digested by XhoI, separated on a 0.9% gel, transferred to Hybond-N+ membrane (Amersham Biosciences), and hybridized to a poly(GT) telomere-specific probe as described previously (35). The GST fusion proteins were overexpressed and purified according to the manufacturer’s instructions. The purified recombinant proteins were analyzed with Coomassie Blue staining, and aliquoted for gel shift assay.

The Gel Shift Assay—Single-stranded DNA oligonucleotides were synthesized and two complementary oligonucleotides were annealed to form a double-stranded DNA, and the substrates were end-labeled with T4 polynucleotide kinase according to the manufacturer’s instructions. The binding reaction mixture (20-μl volume) contained 25 mM Tris (pH 7.9), 150 mM NaCl, 0.05 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1 μg of sperm single-stranded DNA, 1 μg of poly(dC-dI) and the indicated amounts of proteins. Reactions were incubated at 37 °C for 15 min, loaded onto a 5% nondenaturing polyacrylamide gel, and run in a 0.5× TBE buffer. The gel was dried on Whatman paper and imaged with a Typhoon PhosphorImager (Molecular Dynamics).

Gel Filtration—500 μg of purified Deflp-G (aa 381–480) in 100 μl of PBS was loaded on an analytical Superdex 75 column (Amersham Biosciences), which was calibrated according to the vendor’s protocol with protein standard markers including bovine serum albumin (67 kDa), ovalbumin (43 kDa), Chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). The column was developed with PBS at 0.3 ml min−1, and 1-ml fractions were collected. The elution profile was analyzed by Western blot.

RESULTS

Identification of Deflp—To study how telomere replication by conventional replication machinery and telomere elongation by telomerase are connected, an affinity purification of the proteins that interact with Rrm3p was carried out because Rrm3p promotes the replication fork progression through both telomeric and subtelomeric DNA (34). The RRM3 gene was tagged with 13myc epitope, and the yeast whole-cell extract was derived from a strain expressing 13myc-tagged Rrm3p and the yeast whole-cell extract was digested by XhoI, separated on a 0.9% gel, transferred to Hybond-N+ membrane (Amersham Biosciences), and hybridized to a poly(GT) telomere-specific probe as described previously (35).
the same protein independently, in this paper DEF1 (or Def1) instead of RIP1 (Rip1) is used. The DEF1 gene encodes a 738-amino-acid polypeptide with a theoretical and practical molecular mass of 84 and 120 kilodaltons in SDS-PAGE, respectively (38).

To confirm the interaction between Rrm3p and Def1p, co-immunoprecipitation experiments were carried out with both anti-myc and affinity-purified anti-Def1p antibodies, and the immunoprecipitates were analyzed by Western blot with primary antibodies against Def1p and Rrm3p, respectively. As shown in Fig. 1, Def1p was detected in the Rrm3-13myc immunoprecipitates with anti-Def1p antibodies but not from the rrm3 or def1 cells (Fig. 1A); however, we also detect the band that migrates slower than Def1p in the lane marked RRM3-13myc, we thought that it might be a nonspecific protein, which is co-precipitated by myc antibody together with RRM3-13myc complex. Reciprocally, Rrm3p was co-immunoprecipitated with Def1p from the wild-type cells but not from the rrm3 or def1 cells (Fig. 1B). The results from both the affinity chromatography-mass spectrometric analysis and the co-immunoprecipitation experiments suggest that Def1p interacts with Rrm3p.

**Disruption of DEF1 Results in Telomere Shortening**—Before we identified the Def1 function in the telomere, we first performed the telomere Southern blot to detect whether the myc-tagged Rrm3p could complement its function in telomeres in vivo. The genomic DNA from wild-type and RRM3-13myc cells were digested with XhoI, and the telomere length was analyzed by Southern blot hybridization using a specific TG1–3/C1–3A telomeric probe. As shown in Fig. 2A (left panel), the restriction enzyme XhoI cuts yeast DNA in the subtelomeric region, generating a terminal restriction fragment in wild-type cells of ~1.3 kb, ~350 bp of which represents the terminal polyTG1–3/C1–3A tracts. Tagging the RRM3 gene with 13myc has no effect on telomere length, suggesting that RRM3-13myc could function properly in telomeres. Because Rrm3p has been shown to replicate the replication fork progression through subtelomeric and telomeric DNA, and inactivation of Rrm3p has very modest effect on telomere length (34), we wanted to know whether DEF1 has any effect on telomere length regulation. As shown in Fig. 2A (right panel), loss of Def1p resulted in ~1.1 kb of the Y′ telomeric restriction fragments, i.e. ~200-bp shortening of telomeric DNA, in the extensive subcultured cells (~100 generations) (Fig. 2A, right panel). The X telomeres also shortened in response to the def1 mutation (Fig. 2A, right panel). The shortened telomeres were also detected in another strain W303-1A with the deletion of DEF1 (data not shown), suggesting that the positive regulation of Def1p on telomere length is general in *S. cerevisiae*.

Def1p has been reported to interact with Rad26p, which is the *S. cerevisiae* functional homologue of the Cockayne syndrome B gene (CSB/ERCC6) product and belongs to Swi/Snf-like DNA-dependent ATPase (38, 43), and Def1p shares similarity with Snf5p (comprehensive yeast genome data base). Therefore, a possible explanation for the effect of Def1p on telomeres is that Rad26, as well as other SWI/SNF family members, may be involved in telomere maintenance. Def1p has been shown to play a role in the ubiquitinylation and degradation of RNA polymerase II (38), and thus, it is possible that the telomere shortening seen in the def1 cells results from the effect of RNA polymerase II defect. To address these issues, we made several deletion mutants of snf5, rad26, and dst1. DST1 encodes the elongation factor TFIIS, which promotes the readthrough of blocks to elongation by RNA polymerase II (44). The telomere length in snf5, rad26, and dst1 cells was analyzed, and the results are shown in Fig. 2B (wild-type, dst1, rad26, and snf5) indicated that disruption of either RAD26, SNF5, or DST1 has little effect on telomere length regulation. Because Def1p affects the degradation of RNA polymerase II through ubiquitinylation (38), it is possible that deletion of DEF1 affects the transcription or degradation of telomere-binding proteins, e.g. yKu complex, and the telomere phenotype of def1 cells is a secondary consequence. We then examined the level of yKu70 and Rap1p, in both wild-type and def1 cells and did not find any differences by Western blot analysis (Fig. 2C and data not shown), suggesting that telomere shortening of def1 cells was not likely because of the down- or up-regulation of yKu, or Rap1p resulted from a transcription or degradation defect. These data suggest that the function of Def1p in telomeres is separable from its function in other aspects such as DNA repair and RNA polymerase II degradation (38).

**The Slow Growth of def1 Cells Is Not Likely because of Telomere Shortening**—The def1 cells also showed slow growth phenotype as previously reported by Woudstra et al. (38).
noticed that the colony morphology of the \( def1 \) and wild-type were very different (data not shown), and extensive restreaks of \( def1 \) cells gave rise to smaller colonies. We suspected that the slow growth effect of \( def1 \) cells was because of the telomere shortening; however, the slow growth phenotype is not common in the mutations of many other genes that affect telomere replication. The appearance of little colonies of \( def1 \) cells reflects the “petite” phenotype as in the cells with a mutation in \( PIF1 \) gene, caused by the defective maintenance of mitochondrial DNA (36). The yeast cells with certain defect of mitochondria cannot grow on a non-fermentable carbon source such as glycerol. We then examined the growth ability of the \( def1 \) cells on a glycerol plate in which the non-fermentable glycerol was the only carbon source, and the results are shown in Fig. 3A. Like the \( pif1 \) cells, the \( def1 \) cells did not grow to form colonies on the glycerol plate. To detect whether the \( def1 \) cells are devoid of mitochondrial DNA, we stained the \( def1 \) cells with 4',6-diamidino-2-phenylindole (DAPI). The typical image under the fluorescent microscope is shown in Fig. 3B. The punctate staining of mitochondrial DNA seen in the cytoplasmic regions in the wild-type cells disappeared mostly in the \( def1 \) cells. Therefore, the slow growth of \( def1 \) cells was likely because of the mitochondrial defect.

The fact that \( def1 \) cells showed the mitochondrial defect led us to suspect that the telomere shortening seen in the \( def1 \) cells might be the secondary effect of the mitochondrial defect. Therefore, we introduced a \( CEN \) plasmid (pRS316) carrying the \( DEF1 \) gene into the \( def1 \) strain, as shown in Fig. 2A (lanes marked with \( pDEF1 \)). The shorter telomeres were elongated to the wild-type length in the presence of \( DEF1 \) gene. However, the \( def1 \) cells with the \( DEF1 \) plasmid could not grow on the glycerol plate (Fig. 3A, sector of \( pDEF1 \)), because the lost mitochondrial DNA was not be able to recover upon the introduction of the \( DEF1 \) plasmid into the \( def1 \) cells. These results suggested that the telomere shortening effect in \( def1 \) cells was not the secondary consequence caused by mitochondrial DNA loss.

**Def1p Has Function on Telomeres**

**Def1p Genetically Interacts with RRM3**—If \( RAD26 \) and \( RRM3 \) interact with \( DEF1 \), it is possible that \( RAD26 \) or \( RRM3 \) are in the same pathway as the \( DEF1 \) to regulate telomere length. We compared the telomere length in the strains with both single and double mutations (Fig. 4A). As we mentioned before, in \( rad26 \) cells, the telomeres are comparable with that of wild-type cells (Fig. 2B). When both \( RAD26 \) and \( DEF1 \) were deleted, the \( Y^+ \) telomeres shortened to the sizes that were similar to those observed in the \( def1 \) cells (compare lanes marked with \( def1 \), \( rad26 \), and \( def1 rad26 \), suggesting that \( RAD26 \) has no effect on telomere metabolism. Deletion of \( RRM3 \) caused very modest telomere lengthening (~75 bp) most likely because of the replication fork pausing at telomeric regions (34). The deletion of \( DEF1 \) from a strain with the \( rrm3 \) gave rise to the telomeres, which are in the intermediate size between those seen in the \( rrm3 \) and \( def1 \) strain (compare lanes marked with \( rrm3Δ \), \( def1Δ \), and \( def1Δ rrm3Δ \)), suggesting that the way of \( DEF1 \) affecting telomeres might be different from that of \( Rrm3p \). Similarly, deletion of \( DEF1 \) in the \( pif1-m2 \) cells results in shortened telomeres (Fig. 4B, compare lanes \( pif1-m2 \) and \( def1Δ pif1-m2 \)). The observation that deletion of \( DEF1 \) shortened telomeres in wild-type, \( rrm3 \), and \( pif1 \) cells clearly suggested that Def1p plays a positive role in telomere length regulation; however, it was not clear whether or not Def1p regulate telomere length through affecting telomere heterochromatin structure. To answer the question, we performed a telomere position effect assay using a strain in which the \( URA3 \) gene was integrated into the telomere region of the left arm of chromosome VII to address the role of Def1p in telomere silencing (7). For the wild-type cells, the \( URA3 \) gene is subject to telomere position effect (TPE) and transcriptional silencing, and the cells were able to grow either in the presence or absence of 5'-fluoroorotic acid (Fig. 4C). In contrast and as a positive control, the disruption of yKU80, which is required for TPE, led to a loss of TPE and the expression of \( URA3 \), and the cells of \( yku80 \) were not able to grow in the presence of 5'-FOA (Fig. 4C) (45). The disruption of \( DEF1 \) reduced TPE slightly (~10-fold) compared with wild-type strain (Fig. 4C), suggesting that Def1p slightly affects telomere high order structure.

**The Cellular Senescence Was Accelerated in Both def1Δ est2Δ and def1Δ est3Δ Double Mutant Cells**—The observation that
Deletion of DEF1 shortened telomeres in wild-type, rrm3, and pif1 cells clearly suggested that Def1p plays a role in telomere length regulation. To examine whether Def1p is involved in the telomerase recruitment pathway, we then carried out an episomal test to examine the growth phenotype of the strains of wild-type, def1, est3, and def1 est3 with both a spot assay and a serial liquid culture assay as shown in Fig. 5, A and B, respectively. The initial growth of est3 mutant spores was comparable with that of wild-type, and the following ones exhibited senescence (11). The def1Δ est3Δ3 strain could also grow efficiently at the very beginning (e.g., days 1 and 2), but showed more rapid senescence rate than the est3Δ3 strain (days 3 and 4) (Fig. 5B). The telomere Southern blot (Fig. 5C) revealed that the telomeres in the first dilution of def1Δ est3Δ3 cells (lane marked def1Δ est3Δ3 F1) were ~80 bp shorter than that in the first dilution of est3 cells (lane marked est3Δ F1), suggesting that the exacerbated loss of growth ability of def1Δ est3Δ3 cells resulted from the increased telomere-shortening rate, we also detected the same phenotype in the def1Δ est3Δ3 mutant cells (data not shown). These results suggested that Def1p is not involved in the telomerase recruitment pathway.

Def1p Plays a Role in Generating Type II Survivors—To further confirm that Def1p is involved in telomere maintenance, and the telomere shortening seen in def1Δ cells is not a consequence of RNA polymerase II defect (38), we analyzed the survivors that were recovered from est2Δ, est3Δ, def1Δ est2Δ, and def1Δ est3Δ strains. It has been shown that the telomerase-deficient cells can escape replicative senescence to generate survivors by maintaining their telomeres through homologous recombination (46–48). Cells that escape senescence by either amplifying Y′ regions or TG-telomeric repeats to generate type I or type II survivors (42, 46, 47). In the liquid culture experiments, we noticed that during the early postsenescence period, the growth rates of est3Δ3 survivors and wild-type cells were similar but higher than that of the def1Δ est3Δ survivors, and def1Δ est3Δ survivors did not reach the growth levels of wild-type, est3Δ3, or def1Δ single mutants (Fig. 5B, referred to as Cells/ml on the y axis). The recovery time for survivors of def1Δ est3Δ strain appeared to be delayed ~5 days compared with est3Δ3 mutant cells (Fig. 5B, referred as days on x axis). The reason for the slow recovery of def1Δ est3Δ survivors was probably that all of the survivors were type I, because type I survivors grew more slowly than type II survivors (47). We then carried out the telomere Southern blot to analyze which type of survivors were recovered from est2Δ, est3Δ, def1Δ est2Δ, and def1Δ est3Δ senescent cultures, and the results are shown in Fig. 5, C–E. Consistent with a previous report (47), type II survivors (phenotypical amplification of TG-telomeric repeats) were obtained in est2Δ and est3Δ liquid culture because of their faster growth rate compared with type I survivors (lanes marked est2Δ and est3Δ liquid). In contrast, only type I survivors (phenotypical amplification of Y′ repeats) were detected in def1Δ est2Δ and def1Δ est3Δ strains both by cell viability culture experiment and single-colony streak assay (42) (lanes marked def1Δ est3Δ liquid, def1Δ est2Δ liquid and def1Δ est2Δ clones, respectively). These results indicated that Def1p is required for generating type II survivors.

Def1p Interacts with Telomeric DNA Both in Vivo and in Vitro—To further study Def1p, we also attempted to characterize the biochemical properties of Def1p. The purification of full-length of Def1p, which is 738 amino acids long with a molecular mass of 84 kilodaltons, was not successful because we failed to overcome the degradation problem. Instead, the GST-fused truncated forms of Def1p, which are aa 1–207, aa 208–349, aa 350–568, and aa 569–738, respectively, were overexpressed. Among them, the GST-fused aa 1–207 (named Def1p-N) and aa 350–568 (named Def1p-M) were soluble and were purified to near homogeneity (Fig. 6A and data not shown). Because Def1p plays important roles in telomere maintenance, we wanted to know whether Def1p interacts with telomeric DNA tracts in vitro. The abilities of DNA binding for these two purified fragments were analyzed by gel shift assay. We found that the recombinant Def1p-N (aa 1–207), but not the Def1p-M (aa 350–568), could interact with either double-stranded or single-stranded DNA oligonucleotides with telomeric TG1,6/LC1,6A (or TG1,3) sequences (Fig. 6B, lanes a–c, 1–4, and 7–10). The addition of the excess cold oligonucleotides in the reaction mixture competed out the hot probes (Fig. 6B, lanes 5 and 11), whereas the Def1p-M (aa 350–568) did not bind either single- or double-stranded telomeric DNA (Fig. 6B, lanes 6 and 12). Because the function of Def1p is not limited in telomeres, it is possible that Def1p could also interact with...
non-telomeric DNA. As shown in Fig. 6C, Def1p-N could bind double-stranded and single-stranded DNA with random sequences. To characterize whether Def1p has a preference for a GT-rich sequence, we turned to the competitive binding experiment, the result is shown in Fig. 6C (lanes 7–9). The binding of Def1p-N to the single-stranded nonspecific DNA can almost be inhibited by a 2-fold of excess cold single-stranded telomere oligonucleotide, indicating that Def1p prefers the GT-rich sequence in vitro. These results indicated that Def1p binds DNA directly, and the DNA binding domain is within the N-terminal portion, i.e. aa 1–207, of Def1p. These results were also consistent with previous observation that Def1p was purified from the salt-stable chromatin fraction (38).

To find out whether Def1p could specifically interact with telomeric DNA in vivo, a formaldehyde cross-linking experiment was carried out (22). As shown in Fig. 6D, the “TEL” DNA was pulled down by anti-Rap1 antisera (positive control), by the anti-myc antibodies when Def1p was 13myc-tagged, and by anti-Def1p antibodies, indicating that the Def1p interacts with telomeric DNA in vivo. However, both “ADH” and “ARO” DNAs were also immunoprecipitated by Def1p, suggesting that Def1p could also function in other loci than telomeres. The results are consistent with the in vitro data that Def1p could bind both telomeric and non-telomeric DNA (Fig. 6, B and C).

Another striking feature of Def1p is that the glutamine residues occupy 23% of the total sequence. From aa 381 to aa 480, which contains a coil-coil domain of Def1p, 65 residues are glutamines. It is believed that the expansion of glutamine repeats causes eight neurodegenerative diseases, including Huntington disease. In some of these neurodegenerative diseases, e.g. Huntington disease, the proteins with glutamine repeats have been shown to form granular and fibrous deposits.
in the cell nuclei of the affected neurons (49). In vitro, the recombinant 51- and 83-glutamine peptides of Huntington disease protein are able to form amyloid-like fibers (49). Although there are intervals between the segments of glutamines in the glutamine-rich region (aa 381–480) of Def1p, it is possible that the middle part of Def1p, especially the glutamine-rich region, might mediate its oligomerization and thereby facilitate the interaction between DNA and other proteins, which presumably interact with the C-terminal of Def1p. Thus, we overexpressed the GST-fused Def1p-Q, which is the glutamine-rich region (aa 381–480). The GST-Def1p-Q was purified with GST beads (Fig. 6E, left panel, GST-Def1p-Q), the GST tag was digested with thrombin and eliminated with GST beads (Fig. 6E, left panel, After thrombin cut). The purified Def1p-Q was subjected to a gel-filtration column, which had been calibrated with several protein standard markers as indicated in Fig. 6E (right panel), and the elution profile was analyzed by Western blot (Fig. 6E, right panel). The Def1p-Q came out at the fraction 10 where ovalbumin was eluted, corresponding to the molecular size of 43 kilodaltons, indicating that the glutamine-rich region of Def1p could form a trimer or tetramer. Because of the sparsity of the amino group in the Def1p-Q, staining of the polypeptide with Coomassie Blue resulted in the weak signal in the detection (Fig. 6E, left panel, arrow).

**DISCUSSION**

In the present study, we have identified the Def1p and characterized its novel functions in telomeres. The affinity chromatography using Rrm3p-13myc as bait and then one-dimensional liquid chromatography-MS/MS sequencing allowed us to identify the proteins associated with Rrm3p. In our experiments, we found 129, 137, and 127 proteins in the elution of anti-myc antibody affinity column to which the total protein extract from wild-type, rrm3, and RRM3-13myc cells, respectively, was applied. 25 proteins were in the elution of RRM3-13myc sample specifically but not in either wild-type or rrm3 sample, and Def1p was one of the 25 proteins (data not shown). The reasons that we chose Def1p to further our study were as following. 1) Def1p gave a high score that is a main measure-ment of the abundance in the eluate. 2) Def1p was previously identified from the chromatin-associated fraction (38), suggesting that it could interact with DNA directly or indirectly. 3) Def1p, like other telomere proteins, is involved in DNA repair. 4) Def1p interacts with Rad26p, which is an ATPase and belongs to the Swi/Snf family.

The identification of Def1p through its interaction with Rrm3p led us to study the function of Def1p in telomeres. **Def1p** is not an essential gene, and the cells with the **Def1p** deletion are viable but have unstable mitochondrial DNA (Fig. 3) and shorter telomeres (Fig. 2A). Interestingly, it was previously reported that deletion of the **Pif1p** gene of *S. cerevisiae*, which is a homologue of RRM3 and encodes a 5′–3′ DNA helicase, causes mitochondrial defect and telomere elongation (33, 36, 37). Although Def1p genetically and/or physically interacts with both Rrm3p and Pif1p (Fig. 4, A and B), it seems that they are nor in the same pathway to affect telomeres. The Pif1p is a negative regulator and inhibits telomere lengthening through the telomerase pathway, because the elongation of telomeres in the pif1 cell is Tcl1-dependent (33). Rrm3p appears to promote the replication fork to pass through subtelomeric and telomeric regions (34), and the telomere lengthening observed in rrm3Δ cells is likely a secondary effect of replication defects in both subtelomeric and telomeric regions (34).

Def1p is rather a positive regulator in telomere maintenance. The inactivation of Def1p in wild-type, rrm3Δ, or pif1Δ shortens telomeres (Figs. 2A and 4). In addition, the absence of Def1p accelerated telomere-shortening rate in est3Δ cells (Fig. 5C), which caused def1Δ est3Δ double mutant senesced more rapidly than the est3Δ single mutant (Fig. 5, A and B). These results suggested that Def1p is not involved in telomerase recruitment pathway but rather in telomere protection. This argument is supported by the observation that the N-terminal part of Def1p could bind DNA both in vitro and in vivo with telomeric DNA oligonucleotides (Fig. 6, B–D). Although how Def1p is recruited to telomeres remains to be determined, its specific role in telomere maintenance is further strengthened by the finding that Def1p contributed to the formation of type II survivors in the absence of telomerase, because only type I survivors were detected in def1Δ est2Δ and def1Δ est3Δ double mutant strains (Fig. 5, C–E). However, how Def1p participates in the generation of telomerase-independent type II survivors, which involves the RAD50-MRE11-XRS2 complex and RAD52, RAD59, SRS2, SGS1, and TID1 (42, 50), needs further investigation.

The cellular functions of Def1p seem not to be limited in telomeres. Def1p appeared to play a role in mitochondrial DNA maintenance (Figs. 2A and 3), and the influence of Def1p on telomeres is not the secondary consequence of mitochondrial defect. Def1p has been shown to have a function in transcription-coupled DNA repair and in ubiquitinylation of RNA polymerase II and mediating its degradation through its interaction with Rad26p (38). These activities of Def1p seemed to be separable from its roles in telomeres. Neither Dst1p (transcription elongation factor of RNA polymerase II) nor Rad26p has an effect on telomere length regulation because no changes in telomere length were observed in the dst1Δ or rad26Δ cells (Fig. 2B). In addition, the protein level of either yKu or Rap1p did not change in the absence of Def1p (Fig. 2C and data not shown), suggesting that transcription defects, which might cause down-regulation of telomere proteins (such as yKu, Rap1p) or ubiquitinylation deficiency, could cause accumulation of telomere proteins (yKu or Rap1p), not mediate the effects of Def1p on telomeres. These findings further indicated that Def1p has different functions in different cellular processes.

Because Def1p interacts with Rrm3p (Fig. 1), and Rrm3p is required for the normal replication of −1,400 sites (including centromeres, tRNA genes, inactive replication origins, the silent mating type loci, telomeres, and rDNA) (51), it will be interesting to find out whether Def1p affects (or does not) replication fork progression in telomere regions, as well as loci other than telomeres (e.g. rDNA). In addition, the mechanism of telomere replication and maintenance is conserved during evolution, and the DNA damage-dependent ubiquitinylation of RNA polymerase II seen in yeast also occurs in human cells (52). Therefore, it will be appealing to identify the Def1 orthologue in metazoans, including mammals, in the future.

**Acknowledgements**—We thank V. Zakian for strains and plasmids and Y. Tsukamoto and M. P. Hande for advice on yeast genetics and critical reading of the manuscript.

**REFERENCES**

Def1p Has Function on Telomeres