MOLECULAR AND CELLULAR BIOLOGY, Oct. 1998, p. 6121–6130 Vol. 18, No. 10
0270-7306/98/$04.00+0
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Telomere Length Regulation and Telomeric Chromatin Require the Nonsense-Mediated mRNA Decay Pathway

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Received 15 April 1998/Returned for modification 15 June 1998/Accepted 10 July 1998

Rap1p localization factor 4 (*RLF4*) is a *Saccharomyces cerevisiae* gene that was identified in a screen for mutants that affect telomere function and alter the localization of the telomere binding protein Rap1p. In *rlf4* mutants, telomeric silencing is reduced and telomere DNA tracts are shorter, indicating that *RLF4* is required for both the establishment and/or maintenance of telomeric chromatin and for the control of telomere length. In this paper, we demonstrate that *RLF4* is allelic to *NMD2*/*UPF2*, a gene required for the nonsense-mediated mRNA decay (NMD) pathway (Y. Cui, K. W. Hagan, S. Zhang, and S. W. Peltz, Mol. Cell. Biol. 9:423–436, 1995, and F. He and A. Jacobson, Genes Dev. 9:437–454, 1995). The NMD pathway, which requires Nmd2p/Rlf4p together with two other proteins, (Upf1p and Upf3p), targets nonsense messages for degradation in the cytoplasm by the exoribonuclease Xrn1p. Deletion of *UPF1* and *UPF3* caused telomere-associated defects like those caused by *rlf4* mutations, implying that the NMD pathway, rather than an NMD-independent function of Nmd2p/Rlf4p, is required for telomere functions. In addition, telomere length regulation required Xrn1p but not Rat1p, a nuclear exoribonuclease with functional similarity to Xrn1p (A. W. Johnson, Mol. Cell. Biol. 17:6122–6130, 1997). In contrast, telomere-associated defects were not observed in *pan2*, *pan3*, or *pan2 pan3* strains, which are defective in the intrinsic deadenylation-dependent decay of normal (as opposed to nonsense) mRNAs. Thus, loss of the NMD pathway specifically causes defects at telomeres, demonstrating a physiological requirement for the NMD pathway in normal cell functions. We propose a model in which the NMD pathway regulates the levels of specific mRNAs that are important for telomere functions.

Telomeres, the ends of linear chromosomes, have multiple functions within the cell. Telomeres stabilize chromosomes by preventing end-to-end fusions (62, 63, 78, 85). Telomerase, an enzyme necessary for the maintenance of telomeric DNA, is inactive in most somatic cells and is activated in many malignant cells (reviewed in references 3, 8, and 25). Furthermore, the lack of telomerase activity in somatic cells limits the life span of human fibroblasts (5). In addition, genes adjacent to telomeres are often packaged in an inaccessible heterochromatin-like structure (23) that results in epigenetic transcriptional repression termed telomeric silencing or telomere position effect. Telomeric DNA is composed of simple short repeats in most organisms, and telomere length is controlled by the actions of multiple gene products (reviewed in reference 58).

In the yeast *Saccharomyces cerevisiae*, the most abundant telomere-binding protein, repressor activator protein (Rap1p) (9, 53, 80), binds double-stranded telomere repeats and is required for both telomeric silencing (43, 83) and telomere length regulation (59, 61, 83). In wild-type cells, Rap1p localizes to a small number of foci located primarily near the nuclear periphery (21, 38), which colocalize with telomeric DNA (21). A number of mutations, including those in *SIR* genes, result in an altered localization of Rap1p (11) and likely reflect changes in the structure of telomeric chromatin (19, 21).

To identify additional proteins involved in telomere function, we performed a genetic screen using circular plasmids that included both telomeric and centromeric DNA (TEL+CEN plasmids). TEL+CEN plasmids are highly unstable due to antagonism between the telomere and centromere sequences (18). We selected for mutant strains in which this TEL+CEN antagonism was lost (i.e., TEL+CEN plasmids were stabilized). As a secondary screen, we analyzed the nuclear distribution of Rap1p in these mutant strains by indirect immunofluorescence microscopy. To date, we have identified six genes that, when mutated, perturb the localization of Rap1p. These genes are termed Rap1p localization factor (*RLF*) genes (17, 19, 40). The present paper describes the characterization of *RLF4*. In addition to the stabilization of TEL+CEN plasmids and the altered localization of Rap1p, *rlf4* strains are defective in telomeric silencing and have shortened telomeres. Cloning of *RLF4* identified *rlf4-1* as a new allele of *NMD2*/*UPF2*, a gene encoding a component of the pathway required for decay of mRNAs that prematurely terminate translation (12, 28).

This unique nonsense-mediated decay (NMD) pathway targets nonsense messages for rapid degradation. Targets of NMD include mRNAs containing nonsense or frameshift mutations (24, 32, 48, 49, 54, 69), transcripts with short upstream open reading frames (ORFs) (12, 68, 70, 72), and inefficiently spliced, intron-containing RNAs that enter the cytoplasm (29, 32). Nonsense mRNAs are stabilized in cells containing suppressor tRNAs, indicating that NMD targeting is closely linked to premature translation termination (4, 24, 54, 70). The NMD pathway requires three elements: a translational arrest, *cis*-acting sequences within the 5′ proximal two-thirds to three-quarters of the message, and *trans*-acting factors such as the Upf/Nmd proteins. The Upf/Nmd proteins include Upf1p (13, 49, 71), Nmd2p/Upf2p (12, 28), and Upf3p (46, 47, 49). Because Upf1p and Nmd2p, as well as Nmd2p and Upf3p, physically interact (27) and because single and double mutations in any of the *UPF* genes inhibit mRNA decay to the same extent,

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype[a]	Source or reference
W303 Strains		
YJB447	*MAT*α *ade2-1 ura3-1 his3 leu2-3,112 trp1 can1-100* ***VII-L URA3-TEL***	A. Lustig
YJB1274	*Mat*α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* ***nmd2::HIS3***	28
YJB1324	*Mat***a** *ade2-1 his3-11,15* ***leu2****-3,112 trp1-1 ura3-1 can1-100* ***upf1::HIS3***	27
YJB1471	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* ***nmd2::HIS3***	This study
YJB2239	*Mat*α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* ***nmd2::HIS3****/+ est1::URA3/+* ***VR-ADE2-TEL***	This study
YJB2263	*Mat***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* ***VR-ADE2-TEL***	This study
YJB2262	YJB2263 ***nmd2::HIS3***	This study
YJB1680	*MAT*α *ade2-1 ura3-1 leu2-3,112 trp1-1 can1-100* ***VII-L URA3-TEL***	This study
YJB2297	YJB1680 ***upf1::HIS3***	This study
YJB2299	YJB1680 ***nmd2::HIS3***	This study
YJB2739	*MAT***a** *ade2-1 his3-11,15 leu2-3 trp1-1 ura3-1 can1-100*	7
YJB2735	YJB2739 ***pan2::LEU2***	7
YJB2736	YJB2739 ***pan3::HIS3***	7
YJB2740	YJB2739 ***pan2::LEU2 pan3::HIS3***	7
YJB2827	*MAT*α *ade2-1 his3-11,15 leu2-3 trp1-1 ura3-1 can1-100* ***VII-L URA3-TEL***	This study
YJB2823	YJB2827 ***pan2::LEU2***	This study
YJB2824	YJB2827 ***pan3::HIS3***	This study
YJB2826	*YJB2827* ***pan2::LEU2 pan3::HIS3***	This study
S150-2B strains		
YJB276	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3*Δ *ade2*Δ [*cir*$^+$]	S. Enomoto
YJB492	*MAT*α *leu2-3,112 ura3-52* [*trp1-289*][b] *his3*Δ*ade2*Δ *VII-L URA3-TEL* [*cir*^{0}]	S. Enomoto
YJB539	*MAT***a** *leu2-3,112 ura3-52* [*trp1-289*] *his3*Δ *ade2*Δ ***rlf4-1 VII-L URA3-TEL*** [*cir*^{0}]	This study
YJB1222	YJB539 **[p108]**	This study
YJB1260	YJB539 **[pRACUT1]**	This study
YJB1275	YJB1260 **[pRS315-NMD2]**	This study
YJB2541	YJB1260 **[pRS315-NMD2]**	This study
YJB1338	*MAT*α *leu2-3,112 ura3-52* [*trp1-289*] *his3*Δ *ade2*Δ ***rlf4-1 VII-L URA3-TEL*** [*cir*^{0}]	This study
YJB1593	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3*Δ *ade2*Δ *cyh2* [*cir*$^+$] ***nmd2::HIS3*** **[pRACUT1]**	This study
YJB1594	*MAT*α *leu2-3,112 ura3-52 trp1-289 his3*Δ *ade2*Δ *cyh2* [*cir*$^+$] ***nmd2::HIS3*** **[pRACUT1]**	This study
YJB2763	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3*Δ *ade2*Δ *cyh2* [*cir*$^+$] ***nmd2::HIS3***	This study
Other		
YJB2793 (=CH1305)	*MAT***a** *ade2 ade3 leu2 ura3 lys2 can1 Gal*$^+$	42
YJB2794 (=RDKY1977)	*MAT***a** *ade2 ade3 leu2 ura3 lys2 can1 Gal*$^+$ ***xrn1***Δ	34
YJB2796 (=FY23)	*Mat***a** *ura3-52 leu2*Δ*1 trp1*Δ*63*	1
YJB2797 (=DAT1-17)	FY23 *his3*Δ*200* ***rat1-1***	1
YJB2889	*Mat*α *his4-38 SUF1-1 leu2-1,3 trp1-*Δ*1 lys1-1 ura3-52*	46
YJB2890	YJB2889 ***upf3*Δ*1::TRP1***	46
YJB2891	YJB2890 **[pLS17 = pUPF3]**	46
YJB2892	YJB2890 **[pRS316]**	81

[a] Brackets around plasmids indicate that they are extrachromosomal. Boldface indicates relevant genotypes.
[b] Brackets indicate that the mutation is suppressed.

the NMD proteins clearly operate together in the NMD pathway (2, 12).

In the present paper, we demonstrate that *RLF4* is allelic to *NMD2*. Like *nmd2*/*rlf4* strains, both *upf1* and *upf3* strains have telomeric defects. Furthermore, strains lacking Xrn1p, the cytoplasmic exoribonuclease that degrades mRNAs targeted by the NMD pathway, also have short telomeres, suggesting that *NMD* and *UPF* genes affect telomere function via Xrn1p-mediated mRNA decay. Our results indicate that wild-type telomeric chromatin function and telomere length control require the NMD pathway specifically, rather than mRNA decay in general.

MATERIALS AND METHODS

Yeast and *Escherichia coli* strains and plasmids. The genotypes of the yeast strains used in this study are listed in Table 1. *E. coli* XL1-blue (Stratagene, Inc.) was used for all standard plasmid preparations. Yeast cultures were grown overnight at 30°C in either minimal (SDC) or rich (YPAD) medium with 2% glucose, unless otherwise stated.

The plasmid pRACUT1 contains an autonomously replicating sequence (ARS), the *ADE2* gene, centromere sequence from CEN IV, *URA3*, and telomere repeat sequence (18). pRS315-NMD2(X-S) contains the full-length *NMD2* gene on an *Xba*I-*Sal*I fragment (28), cloned into the pRS315 vector backbone (81). *NMD2* was disrupted in the S150-2B background by transforming YJB276 with *Sac*I/*Sal*I-digested pBs-*nmd2::HIS3* (28). This disruption replaces 444 bp of the promoter and 1,286 bp of the coding sequence of *NMD2* with an *Xba*I-*Cla*I fragment containing the *HIS3* gene. Deletion of *NMD2* in YJB276-*nmd2::HIS3* was confirmed by Southern blot hybridization, and original disruptants were back-crossed to YJB1260. Strains YJB1593 and YJB1594 are His$^+$ spores from the resulting diploid.

URA3 was integrated adjacent to the left telomere of chromosome VII at the *ADH4* locus (VII-L URA3-TEL) (23) and was introduced into strains containing *nmd2::HIS*, *upf1::HIS*, *pan2::LEU2*, *pan3::HIS*, and *pan2::LEU2 pan3::HIS3*, all in the W303 background, by standard genetic crosses.

Cloning of *RLF4*. YJB539 (*rlf4-1*) cannot grow on 5-fluoro-orotic acid (5-FOA) due to defective silencing of the integrated *URA3* gene at the left end of chromosome VII (*VII-L URA3-TEL*) (23). YJB539 was transformed (20) with a YCP50-LEU2 library (41), plated onto SDC minus Leu to select for transformants, and then replica plated onto SDC plus 5-FOA to select for restoration of silencing at telomeres. 5-FOA-resistant colonies were then streaked onto SDC minus Leu, SDC minus uracil, and SDC plus 5-FOA plates to identify colonies of cells that contained a library plasmid, the *VII-L URA3-TEL* marker, and were consistently 5-FOA resistant. Of ~125,000 colonies screened, 124 colonies met the criteria described above. Plasmids from these candidates were rescued into *E. coli* (XL1-blue) cells. Plasmid p108 contained an ~11-kb insert and conferred 5-FOA resistance on YJB539.

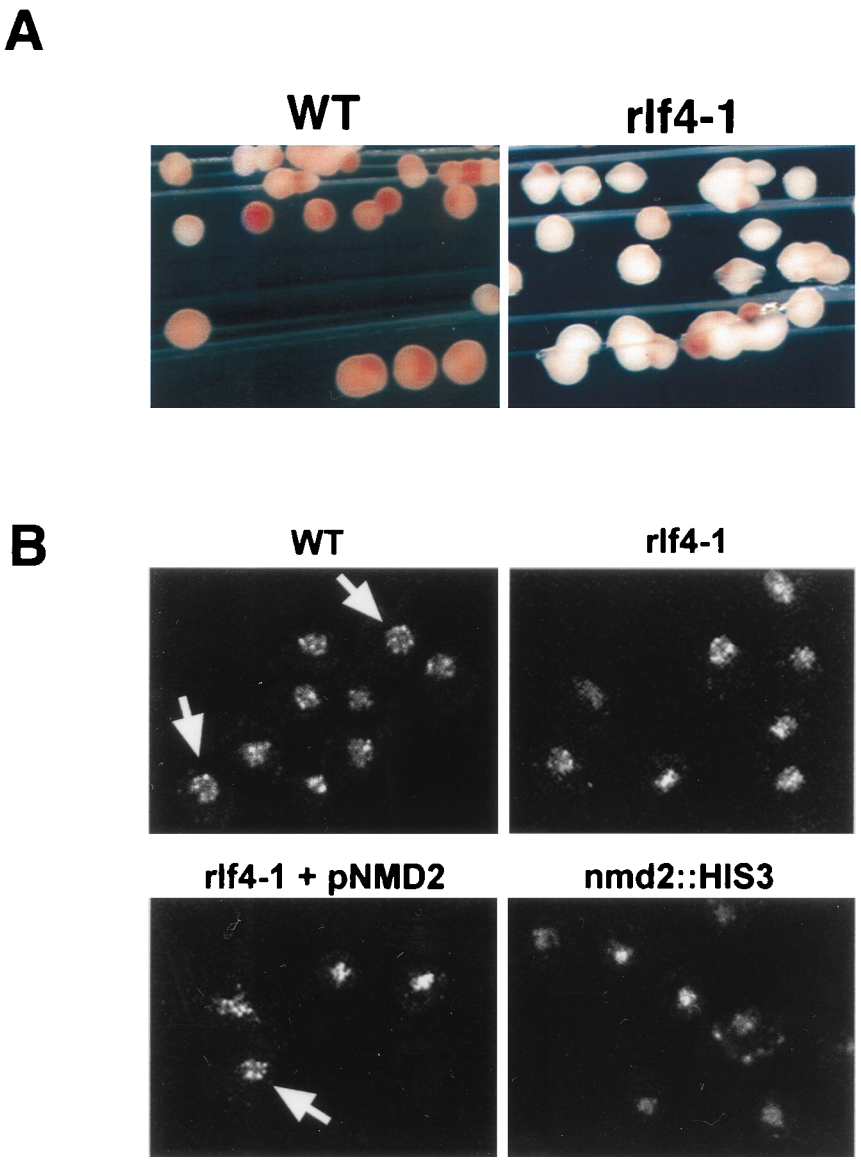

FIG. 1. *rlf4-1* mutant strain exhibits reduced TEL+CEN antagonism and altered localization of Rap1p. (A) TEL+CEN antagonism assay. WT (YJB276) and *rlf4-1* (YJB539) strains were transformed with pRACUT1. The left panel is a transformant of pRACUT1 into YJB276, and the right panel is a transformant of pRACUT1 into YJB539. Transformants were streaked on complete medium to assay loss of pRACUT1, which is indicated by the red. (B) Anti-Rap1p indirect immunofluorescence assay. Images are confocal laser micrographs of the equatorial Z section of each nucleus. WT (YJB492), *rlf4-1* (YJB1260), *nmd2::HIS3* (YJB1274), and *rlf4-1* + pNMD2 (YJB1275) strains are shown. Arrows point to nuclei with characteristic WT Rap1p distribution.

Southern analysis. Telomere repeat probes were prepared by end labeling oligonucleotide 238 (CACCACACCCACACACCACCACACCCACACACCA CCACAC), which is identical to 2.5 repeats of the telomere template sequence in *TLC1*. DNA was denatured and transferred (60) to a nylon membrane (Micron Separations, Inc.). Total genomic DNA was electrophoresed overnight in 1% agarose with Tris-acetate buffer. Blots were hybridized at 65°C in QuikHyb (Stratagene, Inc.) according to the manufacturer's instructions and then washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 65°C for 20 to 30 min and 0.5× SSC–0.1% SDS at 65°C for 10 min.

Northern analysis. Total RNA was prepared from mid-log-phase cultures by a glass bead method (22). RNA was subjected to electrophoresis in 4% formaldehyde–1% agarose gels and transferred (60) to a nylon membrane (Micron Separations, Inc.). *CYH2* probe was prepared by random priming (Oligolabelling kit; Pharmacia, Inc.) of an ~0.5-kb *Eco*RI-*Hin*dIII fragment from pGEM-4Z-CYH2 (28). The blots were incubated with the *CYH2* probe at 42°C in hybridization solution containing 50% formamide, 5× Denhardt's solution, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), 0.1-mg/ml single-stranded herring sperm DNA, 0.2% SDS, and 5.5% dextran sulfate. The blots were washed at 42°C in 2× SSC–0.1% SDS for 20 to 30 min and analyzed on a Molecular Dynamics Storm 840 PhosphorImager with Image Quant version 1.11 software. Ratios were determined by dividing the corrected volumes of pre-mRNA signal by the corrected volumes of mature mRNA signal for each individual strain.

Antisera and indirect immunofluorescence microscopy. Indirect immunofluorescence was performed with anti-Rap1p antiserum exactly as described previously (19).

RESULTS

Telomeric chromatin is altered in *rlf4-1* mutants. In *S. cerevisiae*, telomere repeats, like centromere sequences, stabilize circular plasmids (52). In contrast, circular plasmids containing both telomere and centromere sequences exhibit TEL+CEN antagonism, i.e., they are highly unstable (18). Genes that affect telomeric chromatin (e.g., *RAP1*, *SIR2*, *SIR3*, and *SIR4*)

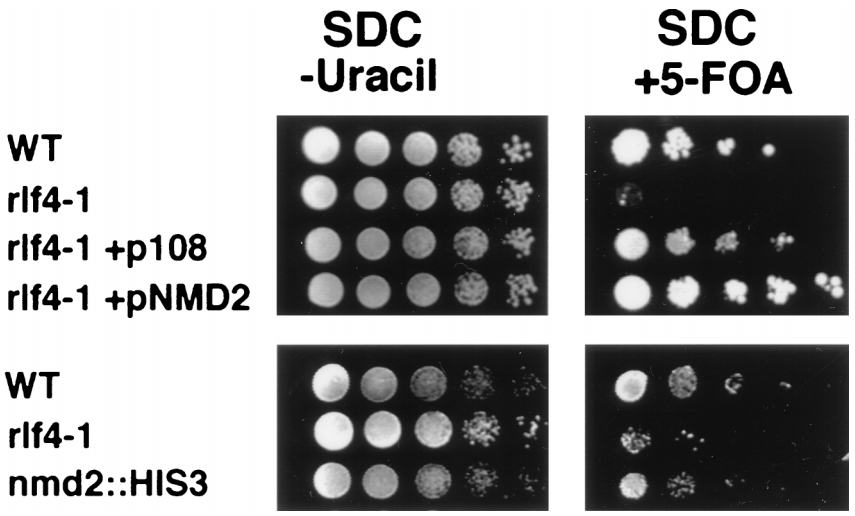

FIG. 2. Telomeric silencing assay. *rlf4-1* mutant strain exhibits reduced telomeric silencing. (Top panels) WT (YJB492), *rlf4-1* (YJB1338), *rlf4-1* + p108 (YJB1222), and *rlf4-1* + pNMD2 (YJB2541); (bottom panels) WT (YJB492), *rlf4-1* (YJB1260), and *nmd2::HIS3* (YJB2763). 1:10 serial dilutions of fresh overnight cultures were spotted onto the indicated media and grown for 48 h at 30°C. Reduced growth on SDC containing 5-FOA indicates reduced silencing of *VII-L URA3-TEL*. Growth on SDC plates (not shown) was similar to growth on SDC plates lacking uracil.

also affect the stability of TEL and TEL+CEN plasmids (18, 52), suggesting that telomeric DNA on plasmids has some of the properties of chromosomal telomeres. The *rlf4-1* mutation was identified in a genetic screen (18, 19) for mutants in which TEL+CEN antagonism was lost, i.e., TEL+CEN plasmids were stabilized. When pRACUT1, a TEL+CEN plasmid carrying *ADE2*, was transformed into an *ade2*Δ strain, the instability of the TEL+CEN plasmid could be visualized by the predominance of red sectors in the colonies (Fig. 1A). In contrast, *ade2*Δ strains carrying the *rlf4-1* allele gave rise to colonies with predominantly white sectors, indicating that the TEL+CEN plasmid was quite stable (i.e., antagonism was lost) (Fig. 1A).

The localization of Rap1p within yeast nuclei, which is thought to reflect the state of telomeric chromatin (19, 21), was altered in *rlf4-1* strains. In wild-type (WT) strains, Rap1p stains as a small number of discrete foci that localize primarily near the nuclear periphery (19, 38) (Fig. 1B). In contrast, in most *rlf4-1* cells, the Rap1p foci appeared less discrete, such that it was difficult to count the exact number of foci within a single nucleus. Diffuse Rap1p staining within the central portion of the nuclei was often evident in *rlf4-1* cells (Fig. 1B). Alterations in Rap1p localization in other strains with defects in telomeric chromatin function have been observed (e.g., *sir3*, *sir4*, and *rlf2* strains) (19, 21).

Genes inserted adjacent to telomeres are silenced in *S. cerevisiae* (23). Telomeric silencing was measured by determining the proportion of cells in the population that repressed a telomeric copy of *URA3* by plating the cells on 5-FOA, which kills cells expressing *URA3* (23). In WT cells, *VII-L URA3-TEL* is subject to epigenetic silencing; more than half of the cells in the population expressed *URA3* and thus could grow on medium lacking uracil; yet a significant proportion (1 to 50%) of the cells also grew on SDC containing 5-FOA, indicating that the *URA3* gene was silenced (Fig. 2). In the *rlf4-1* strain, a very small proportion (10^{-4} to 10^{-5}) of the cells silenced *VII-L URA3-TEL* (Fig. 2). Thus, almost all of the cells were unable to grow on 5-FOA, indicating that *RLF4* is required for telomeric silencing.

Telomere length control is altered in *rlf4-1* mutants. In wild-type *S. cerevisiae* cells, the length of telomere repeat DNA is maintained within a narrow size range (79). Although the average telomere lengths are slightly different in different strain backgrounds, in most strains telomere repeats (TG_{1-3}/$C_{1-3}A$) are ~250 to 350 bp. At least 15 genes that affect average telomere length have been identified (reviewed in references 56, 58, and 86). We measured average telomere length by digesting chromosomal DNA with *Pst*I, which digests ~520 bp from the telomere proximal end of Y′ repeats, which are present at ~50% of the chromosome ends (57). Digestion of the WT strain released an ~800-bp terminal fragment (Fig. 3, arrow), indicating that telomere repeats are an average of ~280 bp in the S150-2B strain background (Fig. 3, lane 1). In *rlf4-1* strains, the terminal *Pst*I fragment was ~700 bp (Fig. 3, lane 2), indicating that the telomere repeat tract was ~100 bp shorter than the telomere repeat tract in the parental strain. An ~100-bp reduction in telomere length was consistently observed in all *rlf4-1* progeny and always cosegregated with the TEL+CEN antagonism and telomeric silencing phenotypes of *rlf4-1* strains. Taken together, the phenotypes of *rlf4-1* strains indicate that both telomeric chromatin function and telomere length control are altered in *rlf4-1* strains.

Cloning of *RLF4*. *RLF4* was cloned by complementation of the telomeric silencing defect in *rlf4-1* strains (see Materials and Methods). One genomic clone, p108, restored TEL+CEN antagonism (data not shown), telomeric silencing (Fig. 2), and WT telomere length (Fig. 3, lane 4) to the *rlf4-1* strain YJB539.

Restriction and DNA sequence analysis of the insert in p108 indicated that the plasmid contained an ~11-kb fragment of chromosome VIII including at least portions of four ORFs: *ORF76W*, *NMD2*, *ORF78W*, and *IRE1*. A plasmid copy of *NMD2* [(pRS315-NMD2(X-S) (28)], which is hereafter called pNMD2, was sufficient to restore TEL+CEN antagonism, telomeric silencing, Rap1p localization, and telomere length control to YJB539 (Fig. 1, 2, and 3; data not shown). Furthermore, *nmd2::HIS3* strains had defects in telomeric chromatin function and telomere length control like those observed in the *rlf4-1* strain; TEL+CEN plasmids were stabilized (data not shown), Rap1p appeared more diffuse (Fig. 1B), telomeric silencing was reduced (Fig. 2), and telomeric DNA was shorter than that in the isogenic WT strain (Fig. 3, lane 3), suggesting

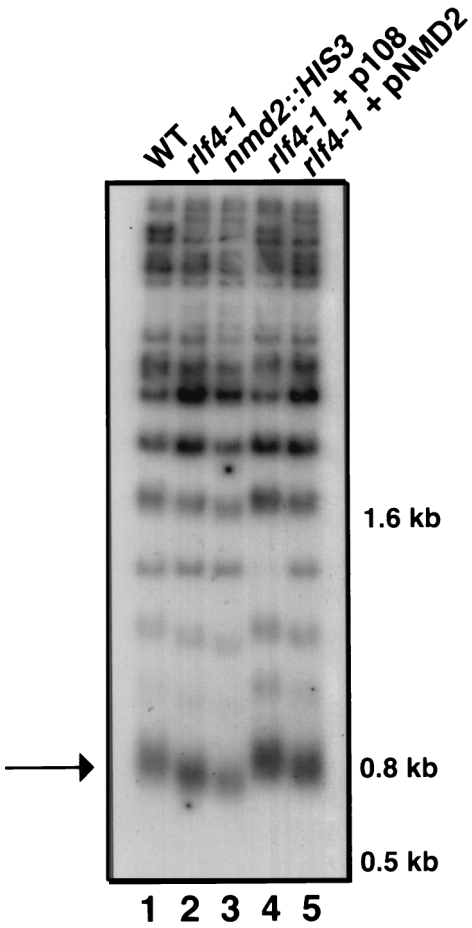

FIG. 3. *rlf4/nmd2* mutants exhibit shortened telomere tracts. Total genomic DNA was digested with *Pst*I and separated in 1% agarose. The arrow points to the terminal *Pst*I fragment of ~800 bp, which includes ~520 bp of the Y′ TAS element and ~180 to 280 bp of telomere repeat sequence $TG_{1-3}/C_{1-3}A$. Other bands are terminal X element fragments and/or internal X and Y′ fragments. Lanes 1 to 5, WT (YJB492), *rlf4-1* (YJB1338), *nmd2::HIS3* (YJB2763), *rlf4-1* + p108 (YJB1222), and *rlf4-1* + pNMD2 (YJB2541), respectively.

that *NMD2* was the gene on p108 responsible for the suppression of *rlf4-1* phenotypes.

Mutations in genes encoding components of the NMD pathway cause steady-state accumulation of a number of RNAs that normally have very short half-lives (reviewed in reference 32). One of these messages is PPR1, a positive regulator of *URA3* (55). Thus, it was possible that derepression of the *VII-L URA3-TEL* in YJB539 occurred due to upregulation by Ppr1p, rather than by reduced telomeric silencing. To distinguish between these possibilities, we monitored the expression of an *ADE2* gene integrated adjacent to the right end of chromosome V (*V-R ADE2-TEL*) (23) in a strain lacking the normal chromosomal copy of *ADE2*. The *ADE2* gene is not upregulated by Prp1p (82) or by mutation of *NMD2* (49a). Red and white colony sectoring was used to monitor the status of the telomeric *ADE2* gene. In these strains, expression of *ADE2* leads to the cells being colored white and lack of *ADE2* expression results in cells being colored red. WT and *nmd2::HIS3* tetrad progenies that carried *V-R-ADE2-TEL* were isolated from the heterozygous diploid YJB2239. WT (*NMD2*) progeny gave rise to red-white sectored colonies (Fig. 4), which are characteristic of the normal epigenetic silencing of telomere-adjacent genes (23). In contrast, *nmd2::HIS3* progeny gave rise to solid white colonies (Fig. 4), which are indicative of extensive derepression of the telomeric *ADE2* gene. Thus, despite the possible upregulation of the *URA3* gene in *nmd2/rlf4* strains, telomeric silencing is perturbed in these strains due to alteration of the telomeric chromatin.

A *rlf4-1* strain is defective in nonsense-mediated mRNA decay. Because the *nmd2::HIS3* strain had defects in telomeric functions similar to those of *rlf4-1* strains, we asked whether an *rlf4-1* strain had defects in nonsense-mediated mRNA decay similar to those of an *nmd2::HIS3* strain. To ensure that genetic background did not contribute to any differences observed, we disrupted *NMD2* in the S150-2B background (see Materials and Methods) and compared mRNA stabilities between isogenic WT, *rlf4-1*, and *nmd2::HIS3* strains.

The *CYH2* transcript is a poorly spliced RNA, and the *CYH2* pre-mRNA accumulates in strains defective in nonsense-mediated mRNA decay (29). We prepared total RNA and asked whether *CYH2* pre-mRNA accumulated in the *rlf4-1* strain as it did in the *nmd2::HIS3* strain by analyzing Northern blots with a *CYH2* probe (Fig. 5). The ratio of *CYH2* pre-mRNA to mature mRNA was approximately twofold higher in the *rlf4-1* strain than the ratio of pre-mRNA to mature mRNA (0.31) in the WT strain (Fig. 5, compare lanes 1 and 2). Introduction of pNMD2 into the *rlf4-1* strain (Fig. 5, lane 4) reduced the ratio of pre-mRNA to mRNA to 0.27, suggesting that the aberrant accumulation of pre-mRNA in the *rlf4-1* strain was due to loss of Nmd2p function.

***rlf4-1* is an allele of *NMD2*.** Since the mutant phenotypes of *rlf4-1* and *nmd2::HIS3* were similar in all cases, and since pNMD2 complemented all phenotypes of *rlf4-1* strains, we performed genetic linkage analysis to ask whether *rlf4-1* and *nmd2::HIS3* were allelic. Haploid *rlf4-1* strain YJB1260 was crossed to haploid *nmd2::HIS3* strain YJB1593, the resulting diploid was sporulated, and TEL+CEN antagonism was scored in the tetrad progeny with pRACUT1. In 10 complete tetrads and more than 60 other individual spores, no restoration of TEL+CEN antagonism was observed, which is consistent with the idea that *rlf4-1* and *nmd2::HIS3* are allelic.

We then determined the nature of the *nmd2* allele from the *rlf4-1* mutant strain by PCR amplification and subsequent DNA sequencing of overlapping segments covering the complete *NMD2* coding sequence. Comparison of the *rlf4-1* allele to the *NMD2* sequence in the GenBank database revealed that the AAG lysine codon at amino acid 304 of *NMD2* had been changed to a UAG stop codon in *rlf4-1*. Thus, the predicted product of *rlf4-1* includes only the N-terminal one-third of the Nmd2p peptide sequence. These data, together with the phenotypic similarities of the *rlf4-1* and *nmd2::HIS3* alleles, indicate that *RLF4* and *NMD2* are allelic.

Telomere functions require the NMD pathway. Nmd2p is a member of the NMD pathway that targets nonsense messages for exoribonucleolytic decay (27, 28). We asked whether the telomeric defects of *rlf4* strains were caused by loss of NMD function or rather by loss of a putative NMD-independent function of Nmd2p. If the telomeric defects of *nmd2* strains are due to inactivation of the NMD pathway, then we expected that mutation of genes encoding other members of this pathway, *UPF1* and *UPF3*, would result in similar telomeric defects.

We generated an *upf1* strain containing *VII-L URA3-TEL* in the W303 background and assayed telomeric silencing in this strain. The silencing defect caused by an *nmd2::HIS3* mutation is less severe in this genetic background (~10- to 100-fold reduction in 5-FOA resistance [Fig. 6A]) than in S150-2B (~100- to 1,000-fold reduction in 5-FOA resistance [Fig. 2]). Similar to an *nmd2::HIS3* strain, the *upf1 VII-L URA3-TEL* strain exhibited an ~100-fold reduction in 5-FOA resistance

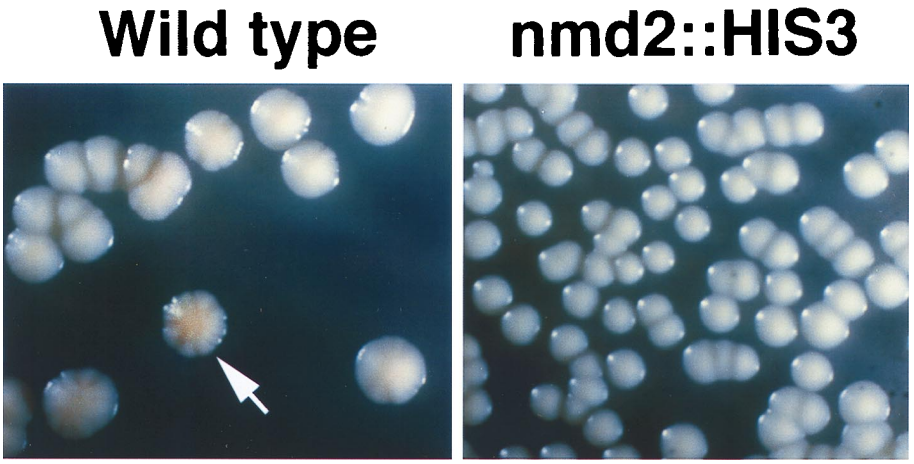

FIG. 4. Silencing of a telomere-proximal *V-R ADE2-TEL* gene is lost in an *nmd2::HIS3* mutant strain. Fresh overnight cultures of WT (YJB2263) and *nmd2::HIS3* (YJB2262) strains were diluted 1:10^5 in sterile water and then plated on complete medium to assay expression of *V-R ADE2-TEL*. Red sectors indicate silencing of *V-R ADE2-TEL*, and white sectors indicate expression of *V-R ADE2-TEL*.

relative to the WT strain, suggesting a modest silencing defect (Fig. 6A). Thus, telomeric chromatin is disrupted to the same degree in *upf1* and *nmd2* strains.

We also assayed telomere length in both an *upf1* strain and an *upf3* strain. Like telomeres in *nmd2* strains, the bulk of the telomeres in the *upf1* and *upf3* strains was ~100 bp shorter than that in the isogenic WT strains (Fig. 6B). *upf1* and *upf3* strains had terminal *Pst*I restriction fragments that were similar in size to those of *nmd2* mutants and were significantly shorter than those of the isogenic WT strains. Addition of plasmid-born *UPF3* to the *upf3* strain restored normal telomere length regulation (Fig. 6B, right panel, lanes 2 and 3). Taken together, the silencing defect in the *upf1* strain and the telomere length reduction in the *upf1* and *upf3* strains suggest that it is the NMD pathway, rather than Nmd2p alone, that contributes to telomere functions in WT cells.

A strain deficient in cytoplasmic exoribonucleolytic activity also has short telomeres. The NMD pathway could contribute to telomere functions by mediating the decay of specific mRNAs. Alternatively, this pathway could perform a novel function at telomeres that is unrelated to its role in mRNA decay. To distinguish between these possibilities, we analyzed average telomere lengths in strains lacking the major exoribonuclease (Xrn1p) required for cytoplasmic RNA decay (44, 65, 67). If the decay of mRNAs targeted by the NMD pathway is responsible for the telomere-associated phenotypes of *upf1*, *nmd2*, and *upf3* mutants, then Xrn1p should also contribute to telomere functions. Consistent with the hypothesis that telomeric defects arise in an *rlf4*/*nmd2* strain due to defective nonsense-mediated mRNA decay, an *xrn1*Δ strain (34) had telomeres ~100 bp shorter than those in the isogenic WT strain (Fig. 7, left panel, lane 2).

Xrn1p, which is exclusively cytoplasmic (30), is functionally similar to Rat1p, a nuclear exoribonuclease that performs an essential function in *S. cerevisiae*. Localization of Rat1p to the cytoplasm allows it to complement an *xrn1*Δ mutation (33, 73). Telomeres are, by definition, nuclear in *S. cerevisiae*, because the nuclear membrane remains intact throughout the cell cycle. Because telomeres are nuclear, we asked whether Rat1p might play a role in telomere function by analyzing telomere length in a *rat1-1* strain. In contrast to the *xrn1*Δ strain, the *rat1-1* strain did not exhibit any significant alteration in the length of the terminal *Pst*I fragment when cells were grown at permissive (23°C) and semipermissive (30 and 33°C) temperatures (Fig. 7 and data not shown). Since Xrn1p and Rat1p can be functionally redundant yet differ in cellular localization (33), the finding that the *xrn1*Δ strain had a defect in telomere length control and that the *rat1-1* strain did not suggests that telomere defects arise due to perturbation of mRNA degradation by a cytoplasmic exoribonuclease rather than due to mRNA degradation by a nuclear exoribonuclease. These results also support the hypothesis that the telomere defects of an *rlf4*/*nmd2* strain occur due to inactivation of the NMD pathway, which targets mRNAs for degradation by Xrn1p.

Telomere functions are not perturbed in strains deficient in the decay of normal messages. Like the NMD pathway, the intrinsic mRNA decay pathway relies on Xrn1p activity for final exoribonucleolytic activity (44, 65, 67). However, the intrinsic mRNA decay pathway requires progressive deadenylation followed by 5′ decapping, whereas the NMD pathway bypasses the deadenylation requirement for mRNA decay (14, 65, 66, 76). Poly(A) nuclease (PAN) deadenylates mRNA in a poly(A)-binding-protein-dependent manner (6, 77). To ask if it is intrinsic mRNA decay or NMD pathway-specific decay that is required for telomere functions, we analyzed *pan2* and *pan3* mutants. *PAN2* and *PAN3* encode two subunits of the PAN nuclease, and *pan2* and *pan3* mutant strains accumulate mRNAs with average poly(A) tails longer than those of WT strains (6,

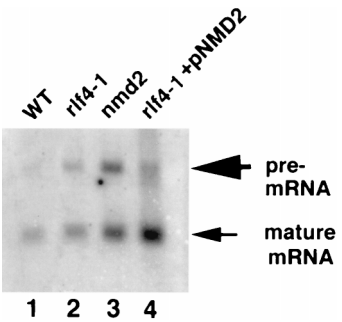

FIG. 5. A *rlf4-1* strain exhibits defective nonsense-mediated mRNA decay. Total RNA was isolated from mid-log-phase cells and separated in 1% agarose–4% formaldehyde. The Northern blot was probed with an ~500 bp radiolabeled fragment of *CHY2* from pGEM-4Z-CYH2.

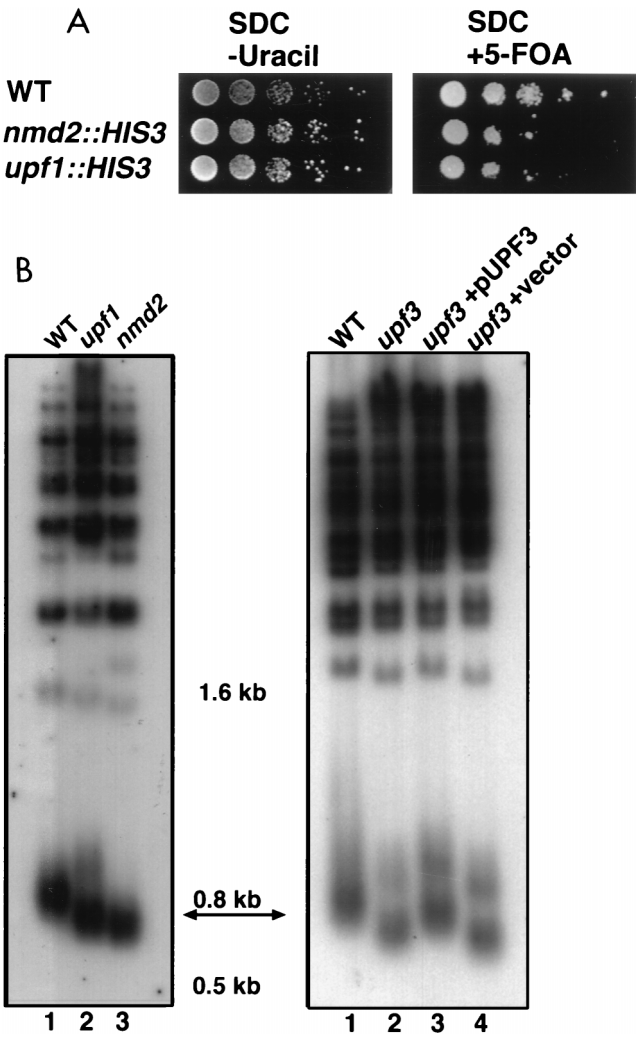

FIG. 6. *upf1* and *upf3* strains exhibit telomeric defects. (A) *upf1* telomeric silencing assay. Serial dilutions (1:10) of fresh overnight cultures were spotted onto the indicated media and grown for 48 h at 30°C. Reduced growth on SDC containing 5-FOA indicates reduced silencing of *VII-L URA3-TEL*. WT (YJB1680), *nmd2::HIS3* (YJB2299), and *upf1::HIS3* (YJB2297) are shown. Growth on SDC plates (not shown) was similar to growth on SDC plates lacking uracil. (B) *upf1* and *upf3* telomere length assay. Total genomic DNA was digested with *Pst*I and separated in 1% agarose. The arrow points to the terminal *Pst*I fragment of ~800 bp, which includes ~520 bp of the Y′ TAS element and ~180 to 280 bp of telomere repeat sequence $TG_{1-3}/C_{1-3}A$. (Left panel) Lanes 1 to 3, WT (YJB447), *upf1::HIS3* (YJB1324), and *nmd2::HIS3* (YJB1274), respectively; (right panel) lanes 1 to 4 WT (YJB2889), *upf3::TRP1* (YJB2890), *upf3::TRP1* + pUPF3 (YJB2891), and *upf3::TRP1* + vector (YJB2892), respectively.

7). We asked whether strains that might be hampered in the processes of normal mRNA turnover would exhibit defects in telomeric functions similar to those of strains deficient in nonsense-mediated mRNA decay. We analyzed telomeric silencing and telomere length control in strains carrying deletions of *PAN2* and *PAN3*. Neither the *pan2* strain, the *pan3* strain, nor a double mutant *pan2 pan3* strain exhibited a reduction in telomeric silencing relative to an isogenic WT strain (Fig. 8A). Similarly, telomere length control in a *pan2* strain, a *pan3* strain, and a *pan2 pan3* double mutant strain was indistinguishable from that of the isogenic WT strain (i.e., terminal telomere tracts were not shortened [Fig. 8B]). Thus, mutants with defects in the deadenylation process required for efficient poly(A) shortening-dependent mRNA turnover do not exhibit altered telomeric chromatin or telomere length control. These results are consistent with the idea that the NMD pathway specifically, rather than the intrinsic pathway that targets mRNA for degradation, is required for normal telomere function.

DISCUSSION

***rlf4-1* is an allele of the nonsense-mediated decay gene *NMD2*.** *RLF4*, which was isolated by virtue of its defects in telomere-associated phenotypes, is allelic with *NMD2/UPF2* by several criteria. First, *NMD2* complemented all of the mutant phenotypes of an *rlf4-1* strain. Second, *nmd2* and *rlf4* strains exhibited similar telomere-associated phenotypes as well as a similar defect in nonsense-mediated decay. Third, genetic linkage analysis indicated that *rlf4-1* and *nmd2::HIS3* cosegregate. Finally, the DNA sequence of *rlf4-1* indicated that it is a nonsense allele that would encode only the first one-third of Nmd2p. The phenotypic similarities of *rlf4-1* and the *nmd2::HIS3* deletion allele suggest that the *rlf4-1* allele is effectively a null allele of *NMD2*.

How does *NMD2/RLF4* affect telomere functions? *NMD2/RLF4* encodes a protein necessary for nonsense-mediated mRNA decay (12, 28). The NMD pathway targets mRNAs for rapid turnover when the translational machinery encounters a premature termination codon during translation (reviewed in references 32, 69, and 70). *NMD2/RLF4* is one of three genes, the other two being *UPF1* and *UPF3*, that are required for nonsense-mediated decay. Like *nmd2/rlf4* strains, *upf1* and *upf3* strains had telomere-associated defects (Fig. 6), suggesting that the NMD pathway, rather than an NMD-independent function of Nmd2/Rlf4p, is required for normal telomere function.

We envision two general models to explain why the NMD pathway proteins might be required for telomere functions: a direct model and an indirect model. In the direct model, NMD proteins have a second function at telomeres in the nucleus. In the indirect model, the NMD pathway affects telomere function by altering the stability of one or more specific mRNAs that are important for telomere chromatin function and/or telomere length control. A number of genes, such as *RAP1* and *RIF1*, affect both telomere length control and telomeric silencing (24a, 59). It is also possible that the NMD pathway affects telomere functions even more indirectly, by altering the level of an mRNA that encodes a protein that then, in turn, regulates telomere-related genes. Nmd2p has a putative bipartite nuclear localization sequence that is required for function (26, 28). Upf3p also has predicted nuclear localization sequences and nuclear export sequences (46, 79a). Thus, at least two of the NMD proteins may have an opportunity to interact directly with telomeres, at least transiently.

To begin to distinguish between the direct and indirect models of NMD protein involvement in telomere function, we asked whether Xrn1p is required for telomere function. *XRN1* has been isolated from a wide range of biochemical and genetic screens and is also known as *KEM1*, *SEP1*, *DST2*, *RAR5*, and *SKI1* (15, 36, 37, 39, 84). We found that, like *upf1*, *nmd2*, and *upf3* mutants, the *xrn1* mutant had short telomeres (Fig. 7, left panel). Our work confirms a previous report that telomeres are shorter in *xrn1/kem1* strains (51). Unlike the previous report, we found that *xrn1* mutants did not display a senescence phenotype (data not shown); rather, in keeping with the observations of others (16, 33, 36, 45), *xrn1* cells exhibited a slow-growth phenotype. The fact that Xrn1p is exclusively cytoplasmic (30, 33) supports the indirect model that Xrn1p (and Upf1p, Nmd2p, and Upf3p) affects telomeres via message degradation.

A second exoribonuclease in *S. cerevisiae* is encoded by the

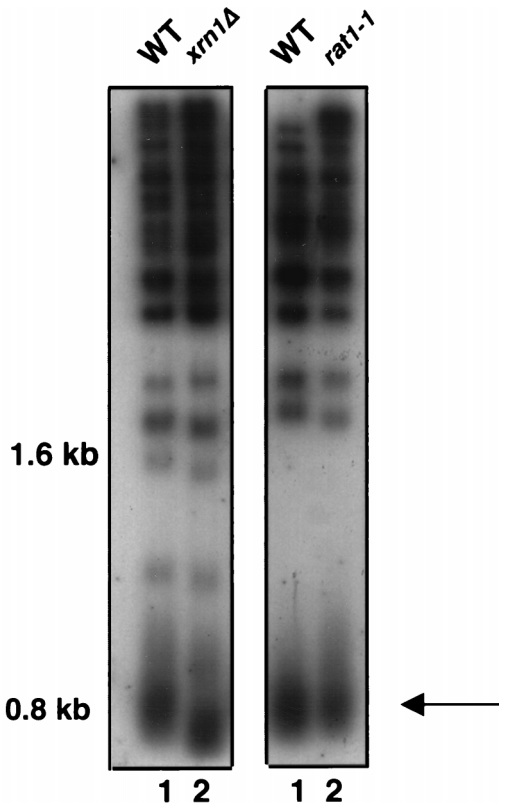

FIG. 7. An *xrn1*Δ strain, but not a *rat1-1* strain, exhibits defective telomere length control. Total genomic DNA was digested with *Pst*I and separated in 1% agarose. The arrow points to the terminal *Pst*I fragment of ~800 bp, which includes ~520 bp of the Y′ TAS element and ~180 to 280 bp of telomere repeat sequence $TG_{1-3}/C_{1-3}A$. (Left panel) Lanes 1 and 2, WT (YJB2793) and *xrn1*Δ (YJB2794), respectively; (right panel), lanes 1 and 2, WT (YJB2796) and *rat1-1* (YJB2797), respectively. All strains were grown at 30°C.

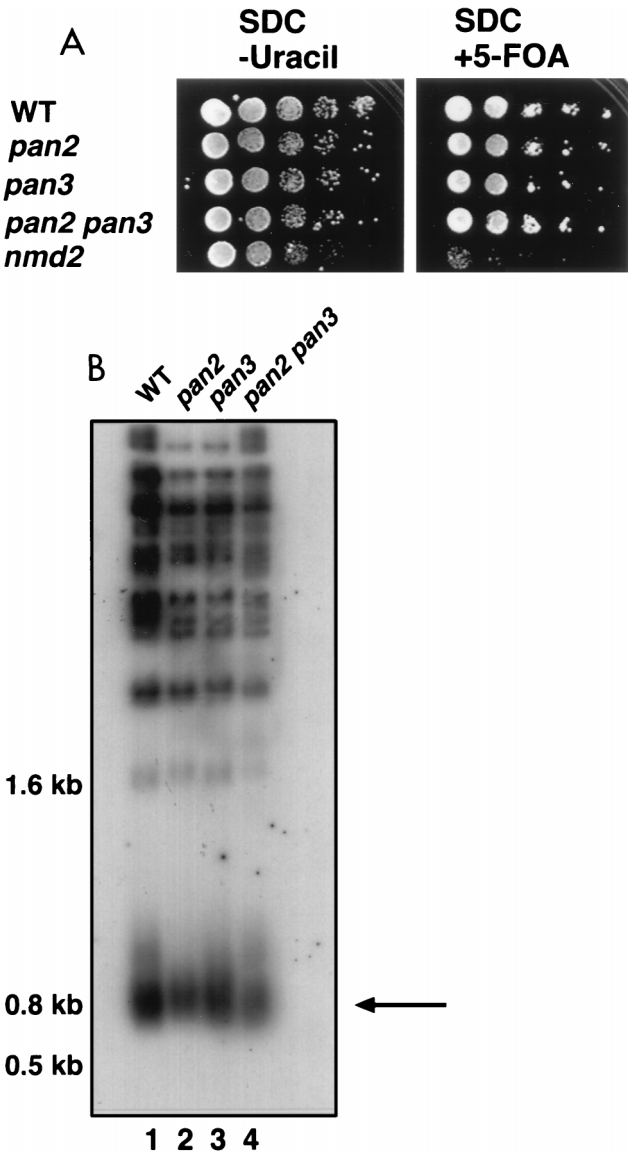

FIG. 8. *pan* mutants do not exhibit telomere defects. (A) Telomeric silencing assay. Serial dilutions (1:10) of fresh overnight cultures were spotted onto the indicated media and grown for 48 h at 30°C. WT (YJB2827), *pan2::LEU2* (YJB2823), *pan3::HIS3* (YJB2824), *pan2::LEU2 pan3::HIS3* (YJB2826), and *nmd2::HIS3* (YJB1593) are shown. Growth on SDC plates (not shown) was similar to growth on SDC plates lacking uracil. (B) Telomere length assay. Total genomic DNA was digested with *Pst*I and separated in 1% agarose. The arrow points to the terminal *Pst*I fragment of ~800 bp, which includes ~520 bp of the Y′ TAS element and ~180 to 280 bp of telomere repeat sequence $TG_{1-3}/C_{1-3}A$. Lanes 1 to 4, WT (YJB2739), *pan2::LEU2* (YJB2735), *pan3::HIS3* (YJB2736), and *pan2::LEU2 pan3::HIS3* (YJB2740), respectively.

essential gene *RAT1* (1, 33, 35). Rat1p localizes predominantly to the nucleus; yet this nuclear localization appears saturable, such that multicopy expression of *RAT1* results in both nuclear and cytoplasmic localization of Rat1p and partially restores the slow growth and RNA turnover phenotypes of an *xrn1*Δ strain (33, 73). In contrast to the *xrn1* mutant, the *rat1-1* mutant had normal telomere length control (Fig. 7), suggesting that, despite the similarities between Xrn1p and Rat1p, the NMD proteins do not affect telomere functions via nuclear Rat1p activity. This result is again consistent with the indirect model that NMD proteins are required for telomere function because they target specific mRNAs for decay by Xrn1p.

Telomere functions could be indirectly affected by all mRNA decay pathways or by the NMD pathway specifically. To distinguish between these possibilities, we analyzed the telomere-associated phenotypes of *pan* strains which are deficient in the poly(A)-binding-protein-dependent PAN. PAN is the only characterized deadenylase in yeast, and poly(A) tail deadenylation is a prerequisite for targeting of a message for the intrinsic pathway of mRNA turnover (6, 7, 14, 77). Pan2p and Pan3p interact physically, and *pan2* and *pan3* mutant strains have mRNA populations with average poly(A) tail lengths longer than those of WT strains (7). The fact that neither a *pan2*, a *pan3*, nor a *pan2 pan3* strain exhibited reduced telomeric silencing or shortened telomere tracts (Fig. 8) is consistent with the hypothesis that it is the NMD pathway specifically, rather than intrinsic mRNA decay, that is required for telomere function. Because some (inefficient) deadenylation occurs in strains lacking Pab1p, it is possible that other Pab1p-independent PANs exist in yeast (7, 10, 76), and, thus, we cannot exclude the possibility that a Pab1p-independent PAN may contribute to telomere function. Despite this caveat, we propose the model that the levels of mRNAs important for telomere functions are inappropriately increased in *upf* and *nmd* mutants, leading to defects in telomeric silencing and length control. This model implies that in WT cells, the level of these mRNAs is regulated by the NMD pathway. While our data support this indirect model, we cannot rule out the pos-

sibility that the NMD proteins have a second, more direct, role in the nucleus that is independent of their cytoplasmic function and of the function of Rat1p and Xrn1p.

Is there a role for the nonsense-mediated decay pathway in regulating normal rather than nonsense mRNAs? Sequence features that the NMD pathway recognizes include upstream ORFs (uORFs) and frameshifts. Interestingly, a number of messages encoding telomere-associated proteins include uORFs or frameshifts. For example, *TEL2*, *EST1*, and *EST2* (genes required for telomerase activity and/or telomere length control) contain clusters of uORFs within 100 bp of the initiation codons (50, 74), and *EST3* requires a frameshift for translation of a full-length functional protein (64). Neither uORFs nor frameshifts alone are sufficient to ensure that mRNAs containing them are targeted by the NMD pathway (71). For example, the *GCN4* mRNA, which is regulated by a mechanism involving four short uORFs, is not targeted for decay by the NMD pathway (reviewed in reference 31). Analysis of specific mRNA levels and mRNA half-lives will be required to determine whether the NMD pathway specifically and directly regulates the expression of genes that encode proteins known to be required for telomeric silencing and/or telomere length control.

The NMD pathway has been presumed to target defective rather than normal mRNAs for degradation (70). The telomere-associated phenotypes of *upf1*, *nmd2*, *upf3*, and *xrn1* mutants implicate the NMD pathway in the regulation of normal mRNAs involved in telomere function. The half-lives of mRNAs are thought to be governed by a number of competing stabilizing and destabilizing forces (reviewed in references 32, 70 and 75). Upf-mediated decay may be one of the destabilizing forces that contribute to the regulation of mRNA half-life for one or more normal mRNAs important for telomere functions.

ACKNOWLEDGMENTS

We thank Maryam Gerami-Nejad for technical assistance and David Gartner and Mark Sanders of the University of Minnesota Imaging Center for assistance with digital imaging. We thank Feng He, Allen Jacobson, Alan Sachs, and Michael Culbertson for providing strains and plasmids. We thank Steve Johnston, Cathy Asleson, Michael Lelivelt, and Jeff Dahlseid for critical reading of the manuscript and many helpful suggestions.

This work was supported by a grant from the National Institutes of Health (GM38636) to J.B.

ADDENDUM IN PROOF

Recently, *CTF13*, a gene important for centromere function, was found to be regulated, indirectly, by the NMD pathway (J. N. Dahlseid, J. Puziss, R. L. Shirley, A. L. Atkin, P. Hieter, and M. R. Culbertson, Genetics, in press).

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