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Yeast Cells Lacking 5′→3′ Exoribonuclease 1 Contain mRNA Species That Are Poly(A) Deficient and Partially Lack the 5′ Cap Structure

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Analysis of the slowed turnover rates of several specific mRNA species and the higher cellular levels of some of these mRNAs in *Saccharomyces cerevisiae* lacking 5′→3′ exoribonuclease 1 (*xrn1* cells) has led to the finding that these yeast contain higher amounts of essentially full-length mRNAs that do not bind to oligo(dT)-cellulose. On the other hand, the length of mRNA poly(A) chains found after pulse-labeling of cells lacking the exoribonuclease, the cellular rate of synthesis of oligo(dT)-bound mRNA, and the initial rate of its deadenylation appeared quite similar to the same measurements in wild-type yeast cells. Examination of the 5′ cap structure status of the poly(A)-deficient mRNAs by comparative analysis of the m^7G content of poly(A)$^-$ and poly(A)$^+$ RNA fractions of wild-type and *xrn1* cells suggested that the *xrn1* poly(A)$^-$ mRNA fraction is low in cap structure content. Further analysis of the 5′ termini by measurements of the rate of 5′→3′ exoribonuclease 1 hydrolysis of specific full-length mRNA species showed that approximately 50% of the *xrn1* poly(A)-deficient mRNA species lack the cap structure. Primer extension analysis of the 5′ terminus of ribosomal protein 51A (*RP51A*) mRNA showed that about 30% of the poly(A)-deficient molecules of the *xrn1* cells are slightly shorter at the 5′ end. The finding of some accumulation of poly(A)-deficient mRNA species partially lacking the cap structure together with the reduction of the rate of mRNA turnover in cells lacking the enzyme suggest a possible role for 5′→3′ exoribonuclease 1 in the mRNA turnover process.

5′→3′ exoribonuclease 1 from *Saccharomyces cerevisiae* has been highly purified and characterized (40, 44). In recent studies of gene cloning and sequencing (21, 22), the enzyme was designated XRN1. The enzyme was found to be a 160-kDa protein acting as a processive exoribonuclease and producing 5′-mononucleotides by a 5′→3′ direction of hydrolysis (40, 44). The mode of hydrolysis by XRN1 was studied in detail by using poly(A), rRNA, and oligo(A) as substrates. Capped mRNA was quite resistant to hydrolysis (39). Poly(I) was not hydrolyzed by the enzyme (44). As a step toward determining the metabolic role of XRN1, the yeast gene designated *XRN1* was cloned and disrupted to determine the effect on yeast cell growth (22). Studies in this laboratory showed that fragments of the internal transcribed spacer 1 of pre-rRNA accumulate in yeast cells lacking the gene and that such yeast cells have an increased doubling time, an increased cell size, and modified protein and mRNA levels (21, 43). The turnover of specific short-lived mRNAs is slowed two- to fourfold, and these mRNAs accumulate (21). Sequencing of the *XRN1* gene showed that it is identical to the yeast *DST2* gene (8) and the yeast *SEP1* gene (46), both demonstrated to encode a protein with DNA strand exchange activity (7, 19), and to the *KEM1* gene (17) and the *RAR5* gene (18), identified on the basis of mutations affecting microtubule function and DNA replication, respectively. Johnson and Kolodner (14) have found that the protein also has low level 5′→3′ DNase activity. A commentary on the gene and its cloning in five laboratories on the basis of different functional characteristics has recently appeared (15). It is difficult to evaluate the significance of the different enzymatic activities found associated with the same protein since studies are still incomplete. We are interested in the protein's role in mRNA metabolism. The purification and characterization of a yeast mRNA-decapping enzyme yielding as products m^7GDP and mRNA chains with 5′-phosphate termini led us to suggest that the two enzymes might be involved in mRNA turnover (41, 42).

In this report, we describe further analysis of mRNA species found in an *XRN1* gene disruptant of *S. cerevisiae*. Special emphasis was placed on analyzing the structure of the 5′ termini of the mRNAs to determine the status of the 5′ cap structure. Essentially full-length mRNA species that are both poly(A) deficient and low in cap structure content are found in the *xrn1* yeast cells. The possibility that such mRNA molecules are intermediates in mRNA turnover is discussed.

MATERIALS AND METHODS

Yeast strains and media. YNN27 (α *ura3-52 trp1-289*) and an *XRN1* gene disruptant (*xrn1* Δ*Bgl*II::*URA3*) derived from it have been described previously (22) and were used in the studies described below. YPD and SD media were as described by Sherman et al. (36).

[^{3}H]adenine labeling. For measurement of [^{3}H]adenine incorporation rates into RNA fractions of YNN27 and *xrn1* cells, the cells were grown in SD medium (100 ml) supplemented with 0.1% Bacto Yeast Extract, 0.2% Bacto Peptone, and 5 μg of adenine per ml. At an A_{650} of 0.40, 0.2 mCi of [^{3}H]adenine (29.2 Ci/mmol; DuPont) was added; 15-ml aliquots of the culture were taken at 30, 60, 90, and 120 min and centrifuged, and the cells were frozen. Radioactivity of fractions bound to oligo(dT)-cellulose was measured after RNA extraction and chromatography as described below.

For measurement of the length of newly synthesized poly(A) tails, wild-type and *xrn1* cells were grown to an A_{650} of 2.0 in SD medium (10 ml) supplemented with 0.25%

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Casamino Acids and labeled by the addition of 25 μl of adenine (2 mg/ml) and 0.15 mCi of [^{3}H]adenine (final specific activity of 0.4 mCi/μmol) for 5 min. The cells were collected by centrifugation, and RNA was extracted as described below. For measurement of the initial deadenylation rates in wild-type and *xrn1* cells, cells were grown in SD medium (35 ml) containing 0.1% Casamino Acids. At A_{650}s of 0.9 (wild-type cells) and 1.7 (*xrn1* cells), [^{3}H]adenine (0.40 mCi; 29.2 Ci/mmol) was added. An 8-ml sample was taken at 5 min, after which thiolutin was added to 3 μg/ml. Samples (8 ml) were then taken at 20, 40, and 80 min and centrifuged, and the cells were frozen. RNA was extracted as described below.

RNA extraction and oligo(dT)-cellulose chromatography. RNA was extracted by a slight modification of the procedure of Domdey et al. (6). The first phenol extraction was carried out for 15 min at 68°C with frequent vortexing. The extracted RNA was dissolved in 10 mM Tris buffer (pH 7.5) containing 1 mM EDTA and 0.2% sodium dodecyl sulfate (SDS). To prepare poly(A)$^-$ and poly(A)$^+$ RNA species, oligo(dT)-cellulose (type II; Collaborative Research Inc.) chromatography was carried out by the procedure of Domdey et al. (6). RNA concentrations were determined by A_{260} measurements.

Northern (RNA) analysis of specific mRNAs. RNA samples were fractionated in 1.5% agarose–2.2 M formaldehyde gels (24) containing 0.23 μg of ethidium bromide per ml. Transfer to nylon membranes (Nytran; Schleicher & Schuell) and immobilization were carried out as described by Maniatis et al. (24). Densitometer scanning of photographic negatives of the ethidium bromide-stained rRNA bands on the gel and membrane was carried out for quantitation of the amount of RNA in each sample. The membranes were hybridized with 5′-^{32}P-labeled oligonucleotide probes (20 to 21 nucleotides [nt]) complementary to mRNA sequences as described by Church and Gilbert (4) and subjected to autoradiography. The probes used were complementary to the following nucleotide sequences of the coding sequence of each gene, shown in the reference cited: *CYC1*, nt 145 to 165 of 330 nt (37); *RP51A*, nt 124 to 144 of 411 nt (45); *PGK1*, nt 159 to 178 of 1,250 nt (13); *ACT1*, nt 1102 to 1122 of 1,125 nt (27); and *MFα1*, nt 350 to 370 of 498 nt (20). The probes were synthesized with an Applied Biosystems 391 DNA synthesizer. The films were scanned with a Molecular Dynamics computing densitometer to quantitate the results, and corrections were made for linearity of both the film and the blots (by analysis of different RNA concentrations).

Measurement of [^{3}H]m^{7}G cap structure. For determination of mRNA cap structure amount, 40-ml cultures of wild-type (A_{650} = 0.56) and *xrn1* (A_{650} = 0.98) cells in SD medium (supplemented with an amino acid mixture [20 μg/ml] lacking methionine) were labeled by the addition of 16 μl of methionine (10 mg/ml) and 1 mCi of [*methyl*-^{3}H]methionine (212 mCi/mmol; final specific activity of 0.93 mCi/μmol; DuPont) for 2 h. RNA was isolated from the cells and subjected to oligo(dT)-cellulose chromatography. The oligo(dT)-unbound fractions were each rechromatographed on a second oligo(dT)-cellulose column to remove all of the poly(A)$^+$ mRNA. To separate small nuclear RNA, the poly(A)$^-$ RNA fractions were then chromatographed on a Bio-Gel A-15m column (Bio-Rad) (1 by 7.5 cm) and eluted with 50 mM Tris-HCl buffer (pH 7.7) containing 100 mM NaCl and 0.2% SDS (Bio-Gel buffer). Fractions collected from 1.9 to 4.7 ml were combined and precipitated with 3 volumes of ethanol. The ethanol precipitates were dissolved in 400 μl of Bio-Gel buffer and chromatographed on a Bio-Gel A-5m column (Bio-Rad) (1 by 7.5 cm) to further separate small nuclear RNA and mRNA. Fractions collected from 2.0 to 4.4 ml were combined, and the RNA was precipitated as just described. These RNA fractions are designated poly(A)$^-$*. The [^{3}H]m^{7}G cap structure was analyzed essentially as described by Sripati et al. (38). The poly(A)$^-$* and poly(A)$^+$ RNA fractions were hydrolyzed with 10 μg of nuclease P1 (Pharmacia) in reaction mixtures (50 μl) containing 20 mM sodium acetate buffer (pH 5.3). After 1 h at 37°C, Tris-HCl buffer (pH 9.0) was added to 80 mM with 1.5 μg of *Escherichia coli* alkaline phosphatase (Sigma). At 40 min, another 1 μg of the same enzyme was added, and incubation was continued for 30 min at 37°C. The reaction mixtures were then applied to Whatman 3MM paper with 5′-GMP as a marker and electrophoresed for 90 min at 2,000 V with pyridine-acetic acid buffer (pH 3.5). The part of each lane corresponding to m^{7}GpppA and m^{7}GpppG spots, the two cap structures found in yeast cells (38) (their positions relative to 5′-GMP were determined in a separate experiment), were eluted with water (1 ml) for 1 h, and the eluates were evaporated to dryness in a vacuum centrifuge. The pellets were dissolved in 50 μl of 50 mM Tris-HCl buffer (pH 7.7) containing 20 mM $MgCl_2$ and incubated for 1 h at 37°C with 0.2 U of venom nucleotide pyrophosphatase (Sigma). *E. coli* alkaline phosphatase (1.5 μg) was then added, and incubation was continued for 1 h at 37°C. The reaction mixtures were then applied to 3MM paper, using m^{7}G (Sigma) as a marker, and electrophoresed as described above. The m^{7}G spots were eluted with water (0.7 ml), and radioactivity was determined.

XRN1 hydrolysis of poly(A)$^-$ and poly(A)$^+$ mRNAs. Poly(A)$^-$ RNA samples (6 μg) and a corresponding amount of poly(A)$^+$ RNAs were dissolved in 50 μl of XRN1 reaction buffer (33 mM Tris-HCl buffer [pH 8.0] containing 2 mM $MgCl_2$, 50 mM NH_4Cl, 0.5 mM dithiothreitol, 5 μg of bovine serum albumin [acetylated albumin; Bethesda Research Laboratories {BRL}], and 40 U of RNasin [Promega]). After 2 min at 37°C, 1 μg of XRN1 (gift from A. Johnson and R. Kolodner, Dana-Farber Cancer Institute; the enzyme was prepared as described in reference 14) was added to the samples designated XRN1+ in Fig. 5. Incubation was continued for the times shown in Fig. 5, and the reactions were then stopped by the addition of 50 μl of 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 0.2% SDS followed by 100 μl of phenol saturated with 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA. After mixing and spinning, the aqueous layers were precipitated with 3 volumes of ethanol. The ethanol precipitates were collected and dissolved in 10 μl of the SDS-containing buffer just described. Aliquots (5 μl) were analyzed by Northern blotting as described above.

Primer extension analysis of mRNA 5′ termini. Oligonucleotides (21 nt) complementary to 5′-terminal sequences of *RP51A* mRNA (as described in Fig. 6) were labeled with ^{32}P at the 5′ termini and hybridized with 30 to 60 μg of total RNA or poly(A)$^-$ RNA and a corresponding amount of poly(A)$^+$ RNA. These procedures and the primer extension analysis were carried out as described by Maniatis et al. (24) with murine leukemia virus reverse transcriptase (BRL). The samples were electrophoresed on 12% polyacrylamide–urea gels (24), using 5′-^{32}P-labeled pBR322 DNA (*Msp*I cut) (New England BioLabs) and 5′-^{32}P-labeled φX174 replicative-form DNA (*Hae*III cut) (BRL) as markers.

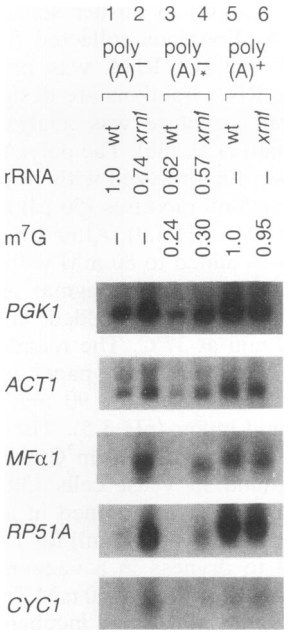


FIG. 1. Poly(A) tail and cap structure status of wild-type and *xrn1* mRNAs. Wild-type (wt) and *xrn1* cells were labeled with [*methyl*-^{3}H]methionine, and RNA was isolated from the cells and processed to obtain poly(A)$^-$, poly(A)$^-$*, and poly(A)$^+$ RNA fractions as described in Materials and Methods. Aliquots (approximately 5%) of each RNA fraction were subjected to Northern blot analysis as described in Materials and Methods for each of the mRNAs shown at the left. At the top are shown the relative amounts of 18S rRNA [wild-type poly(A)$^-$ RNA = 1.0] in the poly(A)$^-$ RNA fractions. The relative levels of cap structure [wild-type poly(A)$^+$ RNA = 1.0] are also shown at the top. The amount of RNA loaded on the gel in the case of wild-type poly(A)$^-$ RNA was 17 μg. The experiment was done in duplicate with similar results, and the calculations are shown in Tables 1 and 2.

TABLE 1. Amounts of poly(A)-deficient mRNAs in wild-type and *xrn1* yeast cells[a]

mRNA	Expt	% Poly(A)$^-$	
		Wild type	*xrn1*
PGK1	1	35	58
	2	37	60
ACT1	1	31	66
	2	32	74
MFα1	1	7	66
	2	10	60
RP51A	1	7	49
	2	17	58

[a] Determined by densitometer scanning of Northern blots as presented in Fig. 1 (lanes 1, 2, 5, and 6) and a duplicate experiment.

RESULTS

Poly(A) tail and total cap structure status of wild-type and *xrn1* mRNAs. Yeast cells lacking an active *XRN1* gene (*xrn1* cells) have an increased size and doubling time. The cells showed significant alterations in protein and mRNA levels, and analysis of mRNA half-life values for several specific mRNAs (mRNAs with diverse function were analyzed) showed that the half-lives of short-lived mRNAs were two to four times longer (21). Two short-lived mRNAs, *CYC1* and *HIS3*, were found to accumulate to cellular levels 2- to 3-fold higher than those of the same mRNAs of wild-type cells, while the levels of the long-lived mRNAs, *PGK1* and *ACT1*, were 1.2- to 1.4-fold higher, possibly the result of the increased size of the *xrn1* cells and longer doubling time (21). To compare both the adenylation and cap structure status of the mRNA species of the wild-type and *xrn1* cells, cells were grown for 2 h with [*methyl*-^{3}H]methionine to label the m^{7}G cap structure. (mRNA of *S. cerevisiae* contains a methyl group only on the cap structure [38].) RNA was isolated and subjected to oligo(dT)-cellulose chromatography, using conditions previously described to bind mRNAs with poly(A) tracts of more than approximately 15 adenylate residues (12). The oligo(dT)-unbound [poly(A)$^-$] and oligo(dT)-bound [poly(A)$^+$] RNA fractions were analyzed by Northern blotting for their content of five mRNA species (Fig. 1). The calculations for this experiment and a duplicate one with four mRNAs are shown in Table 1. Comparison of the wild-type poly(A)$^-$ and poly(A)$^+$ RNAs (Fig. 1, lanes 1 and 5) shows that the *PGK1* and *ACT1* mRNAs were 30 to 35% poly(A)$^-$ (Table 1). The wild-type *MFα1* and *RP51A* mRNAs (short-lived) showed values in the range of 7 to 17% for poly(A)$^-$ RNA (Table 1). The error is higher in the latter determinations because the low mRNA levels found make the Northern blotting much more difficult to quantitate. The *xrn1* poly(A)$^-$ RNA fraction (Fig. 1, lane 2) had a level of each of the mRNAs equal to or greater than that of the *xrn1* poly(A)$^+$ RNA (lane 6). The data in Table 1 show that approximately 60% of the mRNAs of the *xrn1* cells are poly(A) deficient. *CYC1* mRNA of *xrn1* cells was also found to be 60 to 70% poly(A)$^-$ (Fig. 1, lanes 2 and 6), but with the wild-type cells, the level of poly(A)$^-$ species was too low for quantitation in this experiment. Figure 5 shows the relative amounts of CYC1 poly(A)$^-$ mRNA in the wild-type and *xrn1* cells. The experiment of Fig. 1 and the calculations in Table 1 show that the *xrn1* cells contain a higher amount of mRNA molecules that have no poly(A) tails or tails too short to allow oligo(dT)-cellulose binding. Recent results of others (12, 26, 31–35) show that deadenylation of mRNAs may be the first step in the turnover process. These studies are discussed further below.

If XRN1 were involved in mRNA turnover by catalyzing hydrolysis from the 5′ end, removal of the 5′ cap structure of the mRNAs might also be an early step in the turnover process, since XRN1 hydrolyzes capped mRNA slowly if at all (39). To analyze the cap structure content of the poly(A)$^+$ and poly(A)$^-$ mRNA fractions, the poly(A)$^-$ RNA fractions of the [*methyl*-^{3}H]methionine-labeled cells were first subjected to two rounds of chromatography on Bio-Gel columns in order to separate small nuclear RNA, also containing a cap structure, from the mRNA species. The Bio-Gel RNA, called poly(A)$^-$*, and the poly(A)$^+$ RNA fractions were then analyzed for both [^{3}H]m^{7}G content (counts per minute found in m^{7}G after hydrolysis) and the amounts of four mRNAs by Northern blotting. Results of the Northern blots of the poly(A)$^-$* and poly(A)$^+$ mRNA species of both wild-type and *xrn1* cells are shown in Fig. 1, lanes 3 to 6, and the results of the experiment shown there and a duplicate one are presented in Table 2. The amounts (units) of the mRNA species were determined by densitometer scanning of the Northern blots and calculated as described in Table 2, footnote *a*. The values (counts per minute) obtained for the amounts of [^{3}H]m^{7}G cap structure, measured by consecutive hydrolyses of the RNAs with nuclease P1 plus alkaline phosphatase and venom nucleotide pyrophosphatase plus

TABLE 2. m^7G cap structure content of mRNA fractions of wild-type and *xrn1* yeast cells

RNA fraction	Expt	Amt of RNA (U)[a]	Amt of [^{3}H]m^7G (cpm)[b]	cpm/U of RNA
wt poly(A)$^-$*	1	0.18	55	305
	2	0.21	69	328
xrn1 poly(A)$^-$*	1	0.55	69	125
	2	0.65	78	120
wt poly(A)$^+$	1	1.0	231	231
	2	1.0	300	300
xrn1 poly(A)$^+$	1	0.80	219	273
	2	0.95	264	278

[a] Determined by densitometer scanning of Northern blots of four mRNA species, *PGK1*, *ACT1*, *MFα1*, and *RP51A*, as shown in Fig. 1 (lanes 3 to 6). Values are averages of the relative amounts of the four mRNAs, with the poly(A)$^+$ mRNA amount assigned a value of 1.0 in each case.

[b] Measured as described in Materials and Methods. The m^7G content was measured on total mRNA in the fractions, while the amounts of RNA (RNA units) are only for the specific mRNAs listed in footnote *a* (also shown in Fig. 1).

alkaline phosphatase followed by electrophoresis, are also shown in Table 2, along with the counts per minute per unit of mRNA. In Fig. 1, the m^7G values shown above lanes 3 to 6 are relative values [wild-type poly(A)$^+$ mRNA = 1.0]. The results show that the poly(A)$^+$ RNA fractions of the wild-type and *xrn1* cells (Fig. 1, lanes 5 and 6; Table 2) are quite similar in total cap structure content (231 to 300 cpm/unit of RNA). The wild-type poly(A)$^-$* fraction (Fig. 1, lane 3) contained only a low amount of each mRNA, and the m^7G content was 0.24 times that of the poly(A)$^+$ RNA fraction (lane 5). The [^{3}H]m^7G counts per minute per unit of RNA (305 to 328) was quite similar to that of the poly(A)$^+$ mRNA (Table 2). The *xrn1* poly(A)$^-$* RNA fraction (lane 4) was similar to the *xrn1* poly(A)$^-$ RNA fraction in containing considerably more of the mRNA species, amounts almost similar to those of the *xrn1* poly(A)$^+$ fraction in the cases of *PGK1*, *ACT1*, and *MFα1* RNAs. However, this RNA fraction contained only 0.30 times as much [^{3}H]m^7G as did the wild-type poly(A)$^+$ RNA fraction, i.e., only about 125 cpm/unit of RNA (Table 2). The results suggested that the mRNAs accumulated in the *xrn1* cells are partially deficient in both adenylation and cap structure status and led to further analysis of the 5' termini of specific mRNAs as described below.

Sizes of newly synthesized poly(A) tracts; mRNA synthesis and deadenylation rates. The poly(A)-deficient mRNAs of the *xrn1* cells might result from mRNA molecules being synthesized with shorter poly(A) tracts or from a near-normal mRNA deadenylation rate and a slowed mRNA turnover rate due to the absence of 5'→3' exoribonucleolytic activity. It was also possible that the deadenylation reaction is faster in the *xrn1* cells. To determine that long poly(A) chains are formed in the *xrn1* cells, poly(A)$^+$ mRNA of cells labeled with [^{3}H]adenine for 5 min was analyzed for poly(A) chain length by polyacrylamide gel electrophoresis following RNase treatment, essentially according to the procedure of Groner et al. (10). Figure 2A shows the distribution in length of the poly(A) chains of the *xrn1* and wild-type mRNAs after the pulse-labeling. Long poly(A) chains of essentially the same length as wild-type chains are formed in the *xrn1* cells. As measured by the rate of [^{3}H]adenine incorporation, the rate of synthesis of poly(A)$^+$ mRNA in the *xrn1* cells is very similar to that of wild-type cells (Fig. 2B). The data in Fig. 2B can also be used to determine poly(A)$^+$ mRNA decay

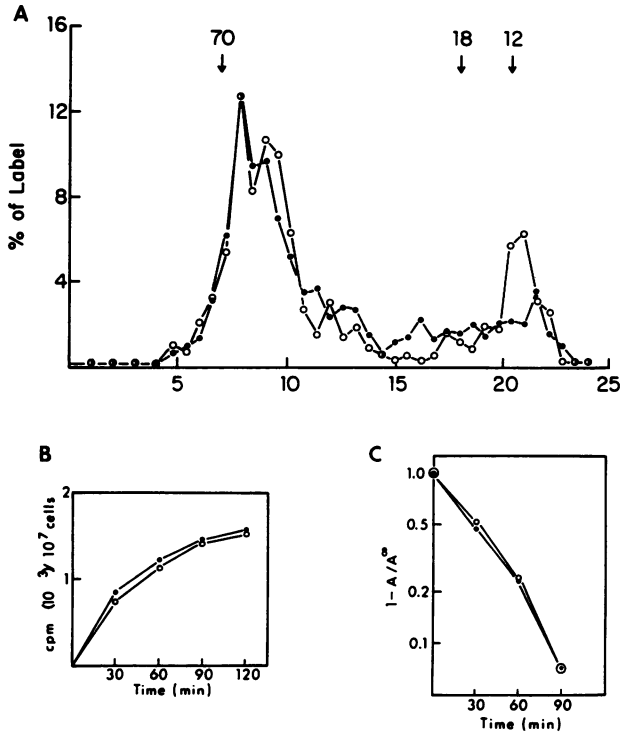


FIG. 2. Pulse-label of poly(A) tracts and poly(A)$^+$ mRNA synthesis and deadenylation rates. (A) Pulse-labeling analysis of poly(A) tail lengths of poly(A)$^+$ mRNA fractions. Wild-type (●) and *xrn1* (○) cells were labeled with [^{3}H]adenine for 5 min, and RNA was extracted as described in Materials and Methods. The RNA was degraded with RNase T_1 (BRL) and RNase A (Sigma) as described by Groner et al. (10) to cleave the RNA except for the poly(A) tracts. The resistant [^{3}H]poly(A) sequences were separated on an oligo(dT)-cellulose column and analyzed on a 15% polyacrylamide sequencing gel (1.5 mm) (24) which was sliced into 1-cm pieces for radioactivity determinations. The slices were incubated in 0.5 ml of 1 N NaOH for 20 h at 37°C. The suspensions were then neutralized, and the amount of ^{3}H label was determined. The results are plotted as the percentage of the total [^{3}H]adenine label in the fractions. tRNA and dA_{18} and dA_{12} oligonucleotides (Pharmacia) were used as markers, and the values in nucleotides are shown at the top. (B) Rates of [^{3}H]adenine incorporation into poly(A)$^+$ mRNA of wild-type (●) and *xrn1* (○) cells. Labeling of the cells, RNA extraction, and oligo(dT)-cellulose chromatography were carried out as described in Materials and Methods. (C) Analysis of poly(A)$^+$ mRNA decay by approach to steady-state labeling. The results shown in panel B were plotted as described by Greenberg (9), i.e., $1 - A/A^\infty$ versus the labeling time (where A is the specific activity of the RNA at the time shown and A^∞ is the specific activity after 120 min of labeling). Symbols: ●, wild-type cells; ○, *xrn1* cells.

rates or, essentially, the half-life of poly(A) tracts, as described by Greenberg (9) and Herrick et al. (12). Log plots of $1 - A/A^\infty$ versus time for the wild-type and *xrn1* cells (Fig. 2C) showed that the average half-lives for the poly(A)$^+$ RNA were approximately 26 and 31 min, respectively. Figure 3 shows an analysis of the poly(A) chain size of poly(A)$^+$ mRNA in the wild-type and *xrn1* cells at different times after addition of the transcription inhibitor thiolutin (29) following a 5-min labeling period with [^{3}H]adenine. Figure 3A represents the 5-min label, at which time thiolutin was added; Fig. 3B to D are analyses at 20, 40, and 80 min, respectively. The rate of initial deadenylation appears quite similar and does not appear to be slowed as is the turnover of the mRNA molecules in the *xrn1* cells.

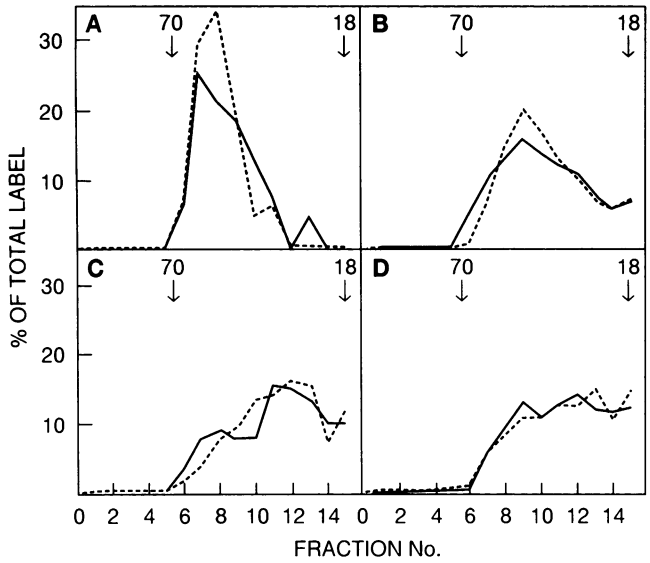
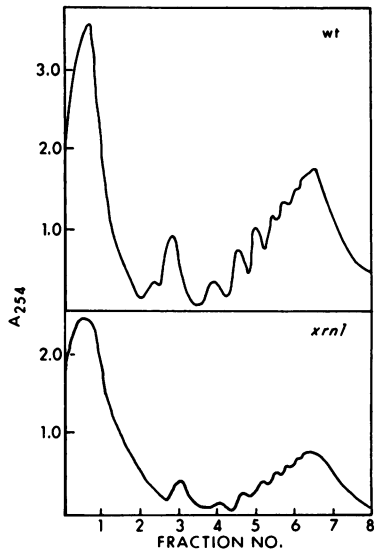


FIG. 3. Time course of change in the mRNA poly(A) tail length after inhibition of transcription by thiolutin. The pulse-labeling, transcription inhibition, and RNA preparation steps in this experiment are described in Materials and Methods. RNA fractions were then treated with RNases, and the poly(A) tracts were isolated and analyzed by gel electrophoresis as described for Fig. 2A. (A) 5 min; (B) 20 min; (C) 40 min; (D) 80 min. Symbols: —, wild-type cells; – – –, *xrn1* cells.



	% of Total						
	1	2	3	4	5	6	7+8
18S rRNA							
wt	-	8	8	7	14	19	44
xrn1	-	6	8	7	10	29	40
ACT1 mRNA							
wt	-	-	-	-	8	28	64
xrn1	-	-	-	-	5	48	47
RP51A mRNA							
wt	-	-	5	12	29	24	30
xrn1	-	-	5	15	34	34	12

FIG. 4. Polysome profiles and mRNA distribution on polysomes. Wild-type (wt) and *xrn1* cells were grown in YPD medium to an A_{650} of 1.5, and extracts were prepared and analyzed as described by Baim et al. (2). One-milliliter aliquots (wild type = 32 A_{260} units; *xrn1* = 22 A_{260} units) were layered on 30-ml sucrose gradients and centrifuged (SW27 head; Beckman ultracentrifuge) for 4 h at 22,000 rpm. Fractions (4 ml) were collected from the top, and RNA was extracted as described by Baim et al. (2). The peak at fraction 3 is the 80S ribosome peak. Northern blot analysis of the RNA fractions was done as described in Materials and Methods. rRNA and mRNA amounts, given as the percentage of the total amount detected in all fractions, were determined by densitometer scanning of the Northern blots and photographic negatives of the gel and membrane. These values for fractions 1 to 8 are shown below the polysome profiles. Fractions 7 and 8 were combined for the RNA extraction, since mixing occurred upon collection of the wild-type gradient.

Polysome analysis. Our previous studies of *xrn1* cells (21) showed that the cellular protein synthesis rate, as measured by [^{3}H]leucine incorporation into protein, is similar to that of wild-type cells. The studies also showed that the rRNA (25S and 18S) content of *xrn1* cells is 0.9 to 1.0 times that of wild-type cells. The doubling time of the *xrn1* cells is increased about twofold, but the cells are, on average, 1.5 to 1.8 times larger. These results suggested that translation is not greatly impaired in the *xrn1* cells. To determine whether the poly(A)-deficient mRNAs of the *xrn1* cells are polysome bound and to examine further a possible translation defect in the *xrn1* cells (a close correlation between the rate of translation and the rate of mRNA turnover in yeast cells has been described elsewhere [12, 31]), polysome profiles of *xrn1* cells and wild-type cells were examined. Polysome profiles obtained with cells from cultures of equal density (A_{650}) are quite similar (Fig. 4), with only a slight reduction in size of the largest polysomes in the *xrn1* cells. (The A_{650} per cell of the *xrn1* cells is 1.8 to 2.0 times that of wild-type cells, so there was less ribosomal material with the *xrn1* preparation). The distribution of mRNA species on the polysomes of the wild-type and *xrn1* cells is as shown by the values (percentage of the total amount detected by scanning of Northern blots) for *ACT1* and *RP51A* mRNAs at the bottom of Fig. 4. In the *xrn1* cells, there is some reduction in the size of polysomes containing the mRNA molecules (about 15 to 20% of the two mRNAs may be shifted to slightly smaller polysomes), but the percentage of mRNA in 80S ribosomes or small polysomes is quite similar to the wild-type value. A duplicate experiment in which the polysome gradients were analyzed as five fractions showed similar results. The changes in polysome profile and mRNA distribution on polysomes in the *xrn1* cells do not appear to be significant enough to support the idea that translation slowing causes the two- to four-times-longer half-lives of specific short-lived mRNAs. The poly(A)-deficient mRNAs of the *xrn1* cells are apparently polysome bound. A recent report by Patel and Butler (30) shows that cells with a temperature-sensitive poly(A) polymerase gene continue translation after a shift to the restrictive temperature, when long poly(A) chains are no longer formed.

Analysis of the 5′ termini of specific mRNAs. As described above, if XRN1 were involved in turnover of the full-length mRNA molecules, decapping or 5′-terminal endonucleolytic cleavage of the RNA would first have to occur, since capped mRNA is quite resistant to hydrolysis (39). The status of the 5′ termini of specific poly(A)$^-$ and poly(A)$^+$ mRNAs of *xrn1* and wild-type cells was examined further in two ways as follows: (i) measurement of the rates of hydrolysis of specific mRNA molecules by highly purified XRN1 and (ii) primer extension analysis of the 5′ termini of *RP51A* mRNA, which has two discrete 5′ termini. The rates of hydrolysis of wild-type and *xrn1* poly(A)$^-$ and poly(A)$^+$ mRNA species

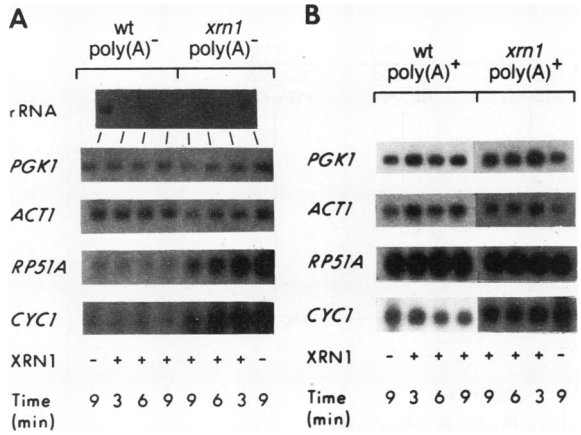


FIG. 5. XRN1 hydrolysis of specific wild-type and *xrn1* poly(A)⁻ and poly(A)⁺ mRNAs. Wild-type (wt) and *xrn1* cells (100 ml) were grown to an A_{650} of 1.5 and collected. RNA was isolated and chromatographed on oligo(dT)-cellulose columns as described in Materials and Methods. The poly(A)⁻ RNA fractions (A) and poly(A)⁺ RNA samples (B) were hydrolyzed with XRN1 (XRN + for times shown) and analyzed by Northern blotting for the specific mRNAs shown as described in Materials and Methods. Control reaction mixtures lacking XRN1 are designated XRN− (9 min). Rapid hydrolysis of the 18S rRNA bands of the poly(A)⁻ RNA fractions, detected by photography of the ethidium bromide-containing gels, occurred and is shown at the top.

were measured by using highly-purified XRN1 (14). The results are shown in Fig. 5. The rapid hydrolysis of the 18S rRNA in the poly(A)⁻ mRNA samples is shown at the top of Fig. 5A. In the case of all four mRNA species tested, the poly(A)⁻ mRNA from the *xrn1* cells was significantly hydrolyzed by XRN1, while the wild-type poly(A)⁻ mRNA was not. Densitometer scanning of the blot showed 30 to 50% hydrolysis of the *xrn1 ACT1* and *PGK1* mRNAs and 50 to 75% hydrolysis of the two short-lived mRNAs, *CYC1* and *RP51A*. The results in Fig. 1 and Table 2 suggested that the poly(A)⁻ mRNA of the *xrn1* cells might be partially deficient in 5′ cap structure. The results here with specific mRNA species support the deficiency of cap structure on essentially full-length mRNA molecules. Poly(A)⁺ mRNA fractions were also examined for their rates of hydrolysis by XRN1 (Fig. 5B). No significant hydrolysis was found for the poly(A)⁺ mRNA species of either wild-type or *xrn1* cells.

To determine whether short sequences as well as the cap structure were removed from the 5′ end of an essentially full-length mRNA of the *xrn1* poly (A)⁻ RNA fraction, poly(A)⁻ and poly(A)⁺ RNA fractions were analyzed by primer extension analysis using oligonucleotides (21 nt long) complementary to 5′-terminal sequences of a short-lived mRNA (*RP51A*). This mRNA was chosen because it has two discrete 5′ termini at nt −19 and −27. Doublet primer extension products at these two sites have been found (45). Three complementary oligonucleotides were used in the primer extension analysis to determine the 5′ termini of the mRNA chains. A diagram of the *RP51A* mRNA 5′ termini and the oligonucleotides used for primer extension with the lengths of the expected products is shown in Fig. 6A. The three oligonucleotides were 171, 114, and 60 nt from the 5′ terminus at nt −27. The object of the experiment was to look for new 5′ ends for the *xrn1* poly(A)⁻ mRNA, and the use of poly(A)⁺ mRNA allowed us to discriminate between nonspecific stops by reverse transcriptase (which should be seen with all the mRNA fractions) and new 5′ ends. The results of the primer extension analyses are shown in Fig. 6B. All three oligonucleotides extended predominantly to the correct termini, and with each gel, the size of the major extension products is shown at the left in nucleotides. Two gels on the primer extension products found with oligonucleotide 171 (oligo 171) are shown as oligo 171-1 and oligo 171-2. The oligo 171-1 gel shows that the oligonucleotide was extended predominantly to positions 170 to 171 and 162 to 163. Arrows 1, 2, and 3, also shown in Fig. 6A, point to three shorter primer extension products found very predominantly (in comparison with the other lanes) with the *xrn1* poly(A)⁻ RNA (called bands 1, 2, and 3). The oligo 171-2 gel was exposed longer so that the wild-type poly(A)⁻ lane shows more clearly, and the results show that band 3 is found only in the *xrn1* poly(A)⁻ lane. Oligos 114 and 60 were used to confirm the finding of shorter primer extension products with the *xrn1* poly(A)⁻ mRNA and to determine their sizes. The oligo 114 gel shows the three shorter extension products, and the oligo 60 gel shows bands 1 and 2. With all three oligonucleotides, band 1, 3 nt short of the terminus at −27 nt, and band 2, 4 nt short of the terminus at −19 nt, were found with the *xrn1* poly(A)⁻ *RP51A* mRNA species. Band 3, about 25 nt shorter, was detectable with oligos 171 and 114. Other shorter bands detected by the analysis and found with all of the mRNA fractions apparently are nonspecific stops or minor 5′ termini. The results with oligos 171 and 114 showed that the new extension products with the *xrn1* poly(A)⁻ mRNA accounted for approximately 30% of extension products with this mRNA fraction. Densitometer scanning of the bands on the oligo 114 gel showed the following percentages for bands 1, 2, and 3 of the total extension products: band 1, 15%; band 2, 13%; and band 3, 9% (total = 37% for the three bands). The results with oligo 60 showed that extension to the final nucleotide was almost completely blocked in the case of the terminus at −19 nt when poly(A)⁻ RNAs were used for the extension analysis (compare lanes 1 and 2 with lanes 3 and 4). The same result was found when a mixture of poly(A)⁻ and poly(A)⁺ RNA or total RNA fractions were used (lanes 5 to 7). With both 5′ termini, the primer extension may be affected by an interaction of the rRNA or other RNA species of the poly(A)⁻ RNA fraction with the positively charged cap structure. The results in lanes 5 to 7 show that bands 1 and 2 are also found with the *xrn1* total RNA fractions, although in the latter case, the bands are a lower percentage of the total extension products since they would be about 15 to 20% of the total mRNA [60% poly(A)⁻ mRNA times 30% shorter products = 18% of total]. As the darker exposure (lanes 5 to 8) at the right of the oligo 60 gel shows, band 2 appears unique and band 1 is very predominant. It cannot be eliminated that more shortened species in the range detectable by the oligonucleotides used for the primer extension are present but too low in concentration to be readily detected.

DISCUSSION

The experiments described in this report show that loss of the yeast *XRN1* gene encoding XRN1 causes the cells to accumulate mRNA species that are both poly(A) deficient and partially lacking the 5′ cap structure. It was previously shown (21, 22) that such yeast cells display increases in cell size (1.5- to 1.8-fold), total protein content, and doubling time but that the rate of protein synthesis measured by [^{3}H]leucine incorporation is nearly normal. The turnover of specific short-lived mRNAs was slowed two- to fourfold,

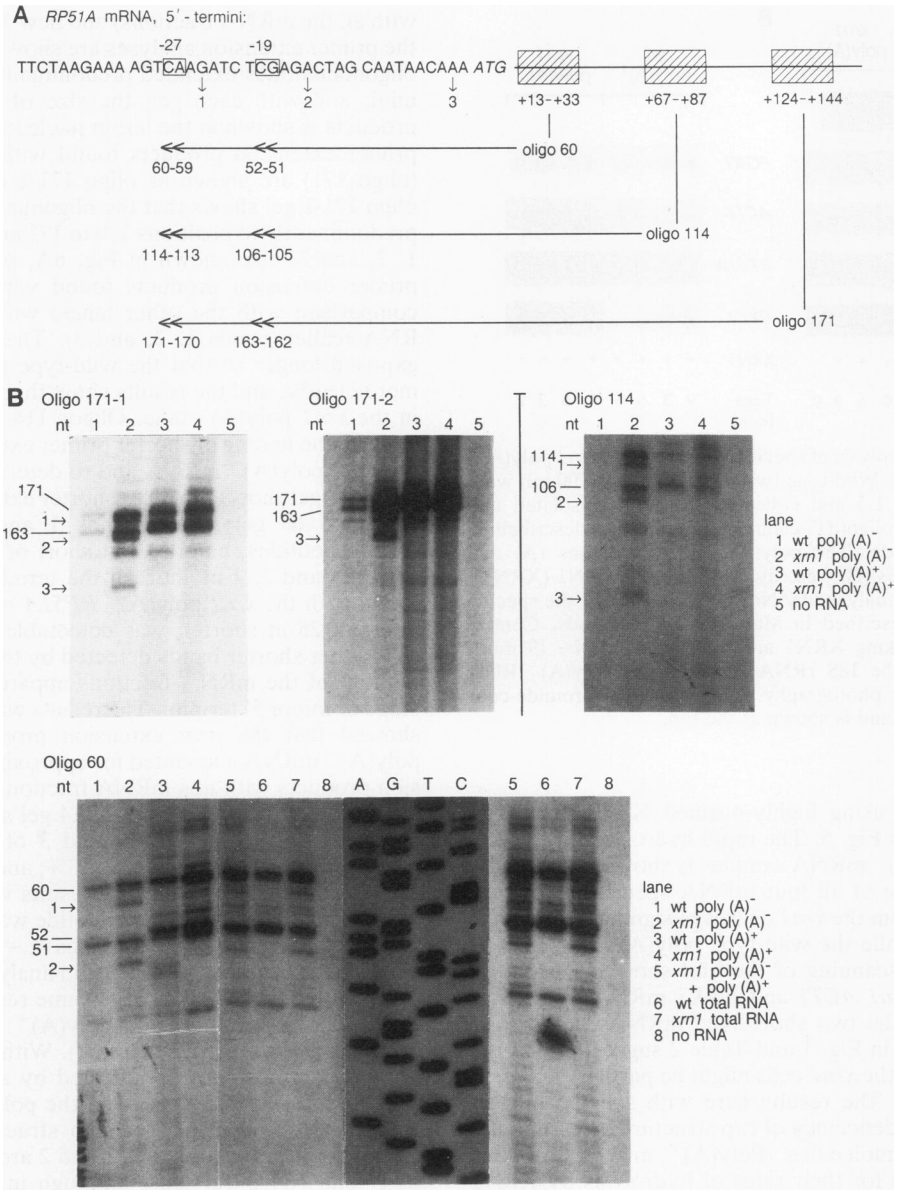


FIG. 6. Primer extension analysis of *RP51A* mRNA 5′ termini. (A) Diagram of the 5′ terminus of *RP51A* mRNA. The 5′-terminal sequence including the transcription initiation sites at nt −27 and −19 is shown (45). Shown to the right of the ATG codon are the three oligonucleotides used for the primer extension. The long horizontal arrows show the lengths of the products expected upon primer extension. The small vertical arrows numbered 1, 2, and 3 denote the 5′ termini of primer extension products (bands 1, 2, and 3) found with *xrn1* poly(A)$^-$ mRNA. (B) Results of the primer extension analysis with oligos 171, 114, and 60. RNA samples prepared as described in Materials and Methods from cells grown to an A_{650} of 1 were used. Two preparations of RNA were used, one for oligos 171 and 114 and a second for oligo 60. The oligonucleotides used as primers were complementary to the sequences shown in panel A, from references given for the oligonucleotide probes in Materials and Methods. Primer extension was carried out as described in Materials and Methods. ^{32}P-labeled *Msp*I-cut pBR322 DNA and *Hae*III-cut ϕX174 replicative-form DNA were used as markers to determine the lengths of the products, indicated at the left. A Sequenase kit (U.S. Biochemical) ladder was run with oligo 60 to determine the sizes of bands 1, 2, and 3, which are also indicated at the left. wt, wild type.

and these mRNAs (*CYC1* and *HIS3*) appeared to accumulate in comparison with the long-lived mRNAs (*PGK1* and *ACT1*) (21). Possibly as a result of the altered mRNA structures and levels, disparate protein levels as analyzed by one-dimensional denaturing polyacrylamide gel electrophoresis were found (21). Perturbations in protein levels could contribute to the altered phenotypes of these cells (reviewed in reference 15). If XRN1 is involved in mRNA turnover by degradation of the full-length mRNA molecules or large fragments, decapping of the 5′ termini would have to occur first, since the exoribonuclease degrades capped mRNA slowly if at all (39). The finding and characterization of an mRNA-decapping enzyme in this laboratory and its possible role in mRNA turnover in conjunction with a 5′→3′ exoribonuclease have been described elsewhere (41, 44). We thus considered it very important to analyze the 5′ termini of the

accumulated poly(A)-deficient mRNA molecules of the *xrn1* cells to determine whether they lacked the cap structure. The poly(A)- and cap structure-deficient mRNAs found here in the *xrn1* cells apparently are formed from normal poly(A)$^+$ mRNAs. There are comparatively more of the short-lived mRNAs, and since the turnover of these mRNAs is slowed more, the results suggest that the deficient molecules may be intermediates in the usual turnover process. However, our results do not eliminate the possibility that the poly(A)- and cap structure-deficient mRNAs are products of faulty mRNA processing.

Studies in other laboratories are pertinent to further discussion of the possible role of XRN1 in mRNA turnover. Yeast mRNA turnover is more amenable to analysis from both a molecular and a genetic approach than is the same process in higher eukaryotes because of the ease of manipulation of yeast cells. mRNA species of yeast cells have half-life values ranging from 1 to over 100 min (3, 12), and progress has been made in an analysis of mRNA sequences that may control stability (3, 11, 28). An analysis of the possible role of yeast destabilizing mRNA sequences is described in a current review (31). Several recent studies (12, 26, 34, 35) suggest that deadenylation of many yeast mRNAs may precede the degradation of the remainder of the chain, as has been found with higher eukaryotic mRNAs (reviewed in references 32 and 33). In 1989, Sachs and Davis (34) reported that depletion of the poly(A)-binding protein (PAB) in *S. cerevisiae* by utilization of promoter inactivation or with a temperature-sensitive mutation in the *PAB1* gene resulted in inhibition of poly(A) tail shortening. A poly(A) nuclease has now been isolated, and its gene has been cloned (35); the results show that it is an essential gene. The enzyme requires PAB for activity, and its degradation of poly(A) tails can be shown to be biphasic (initial and terminal deadenylation). Since a PAB binding site is required for activity, the enzyme initially hydrolyzes poly(A) tracts to lengths of 12 to 25 nt (initial deadenylation). Study of the terminal deadenylation step shows that it may be controlled by destabilizing sequences in the 3′ untranslated region (UTR) of *MFA2* mRNA (23). Results of Muhlrad and Parker (26) also show that mRNA sequences in the 3′ UTR affect the rate of deadenylation which appears to be the first event in the turnover of *MFA2* mRNA. Yeast genes found to affect the turnover process also include the *RNA14* and *RNA15* genes. Mutations in each of the two genes result in an apparent rapid mRNA deadenylation and a fast mRNA decay rate (25).

Overall, nucleases involved in the mRNA decay process have been difficult to identify. Results of Vreken and Raué (48) also suggest a role for a 5′→3′ exoribonuclease. Vreken et al. (49) found that insertion of a G_{18} tract into the 3′ UTR of yeast *PGK1* mRNA increases its half-life by a factor of 2 without affecting its translation. Further studies of Vreken and Raué (48) and Vreken et al. (47) demonstrated that the same insertion causes the accumulation of high levels of a short fragment of the mRNA extending from the G_{18} sequence to the mRNA cleavage-polyadenylation site. Primer extension and S1 nuclease mapping studies showed that the fragment results from an apparent 5′→3′ exoribonucleolytic hydrolysis of short-lived fragments formed by endonucleolytic cleavages close to the 3′ terminus of the coding sequence of the *PGK1* mRNA. Insertion of a G_{18} tract just upstream from the AUG start codon resulted in the accumulation of a 5′-terminal degradation intermediate extending from the site of the G_{18} tract insertion to the approximate proposed sites of endonucleolytic cleavage. The results strongly suggested that a 5′→3′ exoribonuclease impeded by the G_{18} sequences is involved in the turnover of *PGK1* mRNA by degrading endonucleolytically derived fragments.

The results described here on the accumulated mRNAs of the *xrn1* cells being poly(A) deficient, together with the previous finding of slowed mRNA turnover in the cells, are in line with the studies described above on deadenylation being an early event in turnover. As shown in Results, the initial deadenylation process in the *xrn1* cells is slowed little if at all, so one would then expect an accumulation of mRNA molecules lacking long poly(A) tails when further turnover is slowed. The results of Vreken and Raué (48) also suggest that a 5′→3′ exoribonuclease is involved in mRNA turnover; however, their results suggest that its role is in the further hydrolysis of fragments produced by endonuclease action. In our experiments using both oligonucleotide probes and the plasmid DNA probes for *MFα1*, *ACT1*, and *PGK1* described by Herrick et al. (12), no discrete fragments of the mRNAs examined were found on Northern blots even in the turnover experiments previously described (21). Shortening of the larger mRNAs (*PGK1* and *ACT1*) by more than 100 nt would have been detectable by the Northern blotting. In our laboratory, a second enzyme with 5′→3′ exoribonuclease activity has been partially purified from *S. cerevisiae* (22, 40, 41), and a gene with substantial homology to the *XRN1* gene has been cloned on a functional basis in several laboratories (1, 5, 16). The gene encodes a protein of 116 kDa. Results strongly suggest that it encodes the second exoribonuclease. An immunoreactive RNase of 116 kDa is abolished with antiserum against the gene product, and the levels of both the exoribonuclease and the 116-kDa immunoreactive RNase correlate with gene dosage (16). It is possible that the second exoribonuclease partially compensates for loss of XRN1, as we suggested earlier (22). In the case of the results shown here, the degradation found at the 5′ termini of *RP51A* mRNA may result from its action, and subsequent rapid degradation of the mRNA molecules may follow when ribosome loading no longer occurs or after structural impediments are diminished. The second enzyme has not been highly characterized to determine its activity on long RNA substrates. If the second 5′→3′ exoribonuclease can replace XRN1 but is also impeded by G_{18} sequences, it might explain why Vreken and Raué found fragments and that we did not. If either full-length mRNA molecules or large fragments are degraded by XRN1, cap structure removal would have to occur first as described above. The full-length altered mRNA molecules described here in the *xrn1* cells may then be intermediates usually further hydrolyzed by XRN1 and more slowly by a second exoribonuclease or products that arise as a result of a slowed endonucleolytic activity. It is possible that an endonuclease and XRN1 are both part of a turnover complex. The absence of XRN1 may result in a less active degradation complex and the production of the mRNA molecules described here in the *xrn1* cells.

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