Deadenylation of the unstable mRNA encoded by the yeast *MFA2* gene leads to decapping followed by 5' → 3' digestion of the transcript

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The first step in the decay of some eukaryotic mRNAs is the shortening of the poly(A) tail. To examine how the transcript body was degraded after deadenylation, we followed the decay of a pulse of newly synthesized *MFA2* transcripts while utilizing two strategies to trap intermediates in the degradation pathway. First, we inserted strong RNA secondary structures, which can slow exonucleolytic digestion and thereby trap decay intermediates, into the *MFA2* 5' UTR. Following deadenylation, fragments of the *MFA2* mRNA trimmed from the 5' end to the site of secondary structure accumulated as full-length mRNA levels decreased. In addition, in cells deleted for the *XRN1* gene, which encodes a major 5' to 3' exonuclease in yeast, the *MFA2* transcript is deadenylated normally but persists as a full-length mRNA lacking the 5' cap structure. These results define a mRNA decay pathway in which deadenylation leads to decapping of the mRNA followed by 5' → 3' exonucleolytic degradation of the transcript body. Because the poly(A) tail and the cap structure are found on essentially all mRNAs, this pathway could be a general mechanism for the decay of many eukaryotic transcripts.

Differences between the decay rates of mRNAs, as well as changes in the rate of decay of individual transcripts, can significantly affect the level of gene expression in eukaryotes. To understand how these differences in mRNA decay rates are produced and regulated, it is critical to define the mechanisms of mRNA degradation. One major mechanism of mRNA decay, observed for eukaryotes as diverse as mammals and yeast, is initiated by the cytoplasmic shortening of the poly(A) tail (Brewer and Ross 1988; Wilson and Triesman 1988; Swartwout and Kinniburgh 1989; Laird-Offringa et al. 1990; Shyu et al. 1991; Muhlrad and Parker 1992; Decker and Parker 1993). We have provided evidence in yeast cells that shortening of the poly(A) tail leads to 5' → 3' degradation of at least the 3' portion of the transcript body for both the stable *PGK1* ($t_{1/2} = 45$ min) and unstable *MFA2* ($t_{1/2} = 3.5$ min) mRNAs (Decker and Parker 1993). The key observation supporting this conclusion is that when strong RNA secondary structures are inserted into the 3'-untranslated region (UTR) to slow exonucleases, mRNA fragments corresponding to the portion of the *PGK1* and *MFA2* mRNAs 3' of the secondary structure

accumulate after deadenylation (Decker and Parker 1993). This result indicates that the nucleolytic events triggered by deadenylation are 5' of the 3' UTR.

There are two general types of mechanisms by which deadenylation could lead to 5' → 3' degradation of the 3' portion of an mRNA. First, deadenylation could lead to endonucleolytic cleavage at specific sites within the coding region, thus exposing the resulting 3' fragment to 5' → 3' exonuclease digestion. Alternatively, because the 5' cap structure can protect mRNAs from 5' → 3' exonucleases (Furuichi et al. 1977; McCrae and Woodland 1981), deadenylation could lead to the removal of the 5' cap allowing 5' → 3' digestion of the entire transcript body. Because in either case the transcript would be degraded in a 5' → 3' direction, the examination of the structure of the mRNA and any possible decay intermediates under conditions that block 5' → 3' exonuclease action should identify the sites of nucleolytic cleavage following deadenylation. Two experiments of this nature have been done and provide contradictory results. In one experiment, a strong RNA secondary structure was inserted into the 5' UTR of the stable *PGK1* mRNA to physically block exonuclease digestion. This insertion led to the accumulation of an mRNA fragment lacking the 3' portion of the mRNA and was interpreted to indicate that the *PGK1* mRNA was endonu-

cleolytically cleaved within the coding region (Vreken and Raue 1992). Conversely, yeast strains deleted for the *XRN1* gene, which encodes a major 5' → 3' exonuclease, accumulate some mRNAs, including the *PGK1* transcript, as poly(A)-deficient transcripts that are apparently full length. Because a portion of these poly(A)-deficient transcripts can be degraded in vitro by an exogenously added 5' → 3' exonuclease, these transcripts are inferred to be lacking a cap structure (Hsu and Stevens 1993). These data raise the possibility that deadenylation leads to a nucleolytic cleavage event near the 5' terminus.

A major limitation in both of the above experiments is that these studies were confined to the analysis of steady-state populations of transcripts and therefore could not establish precursor–product relationships. Thus, it was not possible to determine whether the observed mRNA species represent true intermediates in the decay pathway or were produced by an alternative mechanism. For example, the *PGK1* mRNA fragment lacking the 3' end that accumulated when the secondary structure was inserted in the 5'-UTR may be produced only when the normal pathway of 5' → 3' degradation is blocked. Similarly, it could not be determined whether the poly(A)-deficient, capless mRNAs identified in *xrn1Δ* cells were decapped prior to deadenylation or were the result of a decapping reaction that is activated by deadenylation. Given these limitations, to determine the nucleolytic events that occur after deadenylation it will be necessary to examine the mRNA species that accumulate when 5' → 3' exonuclease digestion is blocked under conditions where a precursor–product relationship with the starting mRNA can be established. Previously, we described a method utilizing the carbon source regulation of the yeast *GAL1* UAS to rapidly induce, and then quickly repress, transcription of a yeast gene (Decker and Parker 1993). This type of experiment (referred to as a transcriptional pulse–chase) creates a pulse of transcripts whose decay can be monitored during an ensuing chase and allows the determination of precursor–product relationships.

In this work we examined the decay of the *MFA2* mRNA to identify the nucleolytic events triggered by deadenylation. We have chosen to examine the *MFA2* mRNA because deadenylation is the first step in its degradation (Muhlrad and Parker 1992; Decker and Parker 1993), and its small size (~330 nucleotides) simplifies the analysis of poly(A) tail lengths and mRNA structure. We followed the decay of the yeast *MFA2* transcript utilizing either the insertion of strong RNA secondary structures or the inactivation of the *XRN1* gene to block 5' → 3' exonuclease function. The structure of the mRNA species that showed a precursor–product relationship with the full-length mRNA indicated that deadenylation of the *MFA2* transcript leads to decapping and thereby exposes the transcript to the 5' → 3' exoribonuclease encoded by the *XRN1* gene. These results describe the set of nucleolytic events necessary to degrade the entire *MFA2* mRNA. In addition, these observations imply that an interaction exists between the 5' and 3' ends of eukaryotic transcripts.

Results

The first approach we used to determine whether deadenylation leads to decapping or endonucleolytic cleavage was to examine the decay of *MFA2* transcripts that contained strong RNA structures in their 5' UTRs. As shown in Figure 1A, we inserted an 18-nucleotide run of guanosines at the fifth, twelfth, or twenty-eighth nucleotide of the *MFA2* 5' UTR. The poly(G) tract forms an extremely stable RNA secondary structure (Zimmerman et al. 1975; Williamson et al. 1985) that is an efficient structural block to yeast 5' → 3' exonucleases (Vreken and Raue 1992; Decker and Parker 1993). We then examined the decay of these transcripts utilizing the transcriptional pulse–chase procedure. If deadenylation triggers cleavage at, or near, the 5' end we would expect mRNA fragments to accumulate that are shortened from the 5' end to the site of the secondary structure insertion.

For comparison, the analysis of the wild-type *MFA2* transcript by a transcriptional pulse–chase is shown in Figure 1B (top). After a pulse of transcription (0 min lane) the mRNAs have long, relatively homogeneous poly(A) tails [as judged by comparison to a sample in which the poly(A) tails have been removed by treatment with oligo(dT) and RNase H (0dT lane)]. These poly(A) tails then shorten with time, in a somewhat heterogeneous manner, to produce transcripts with short oligo(A) tails. Decay of the body of the *MFA2* transcript initiates once deadenylation to this oligo(A) tail length has occurred (Decker and Parker 1993).

Two important observations are seen in the analysis of the transcripts containing the poly(G) tract within the 5' UTR. First, the decay rate and the deadenylation rate of the resulting mRNAs are unaffected as compared with the wild-type transcript (Fig. 1B,C). Moreover, as seen with the wild-type *MFA2* transcripts, there was a temporal lag following transcriptional repression before decay of the poly(G)-containing transcripts initiated. This lag corresponded to the time it took for a significant portion of the *MFA2* transcripts to be deadenylated to an oligo(A) form (Fig. 1C). This observation suggested that the poly(G)-containing transcripts still require deadenylation before decay. This conclusion is supported by the observation that decay intermediates (see below) began to appear only after a substantial fraction of the mRNA had been deadenylated and that these intermediates had oligo(A) tails, even at times when the mRNA population consisted of transcripts with both long and short poly(A) tails (Fig. 1B). These results indicated that the insertion of the poly(G) tract did not alter the normal mechanism of degradation. Since the presence of secondary structures in the 5' UTR can inhibit translational initiation (Cigan et al. 1988; Van den Heuvel 1990; Abastado et al. 1991; Laso et al. 1993), we examined the translation of these poly(G)-containing transcripts both by immunoprecipitating the MFA2 protein following pulse labeling and assaying fusions to the *lacZ* reporter gene for β-galactosidase activity (e.g., Beelman and Parker 1994). As judged by these assays the poly(G) insertion in the 5'
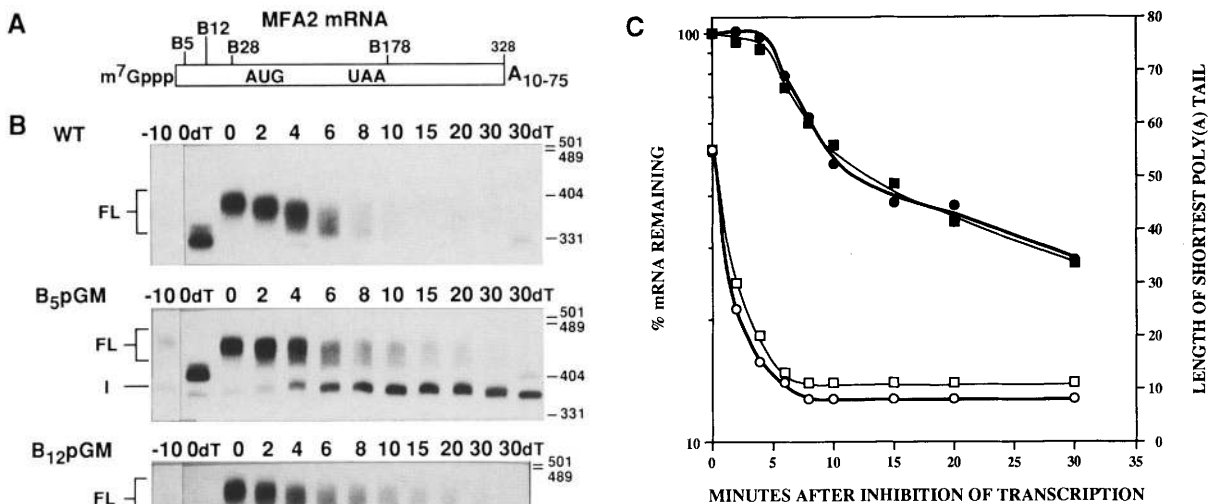
Figure 1. Insertion of a strong RNA secondary structure into the 5′ UTR of *MFA2* mRNA traps decay intermediates. (*A*) The positions of the *Bgl*II sites introduced by oligomutagenesis (see Materials and methods) into the *MFA2* transcript, which have no effect on mRNA decay (data not shown), are shown schematically. Each *Bgl*II site is indicated by a B and the position of the first nucleotide in the restriction site relative to the major 5′ end of the mRNA. An oligonucleotide containing a poly(G) tract was then inserted into these sites (see Materials and methods). (*B*) Northern blots of 6% polyacrylamide/8 M urea gels examining the decay of *MFA2* transcripts with or without (WT) poly(G) insertions in the 5′ UTR by transcriptional pulse–chase (see Materials and methods) in the yeast strain yRP582. The transcripts with poly(G) insertions are designated by the position of the *Bgl*II restriction site used to introduce the poly(G) sequence and pG to denote the insertion. In all panels, lanes are labeled as follows: (−10) RNA prepared from cells immediately after addition of galactose; (0, 2, 4, 6, 8, 10, 15, 20, 30, 40, 60, 80) minutes after transcriptional repression; (0dT and 30dT) samples from these time points that have been cleaved with RNase H and oligo(dT) to remove the poly(A) tail. (FL)Full-length transcripts with poly(A) tails of various lengths; (I) The positions of decay intermediates trimmed at the 5′ end. The probe was either a random-primed probe containing the entire *MFA2* transcript (for the wild-type *MFA2* mRNA) or an oligonucleotide, oRP121 (5′-AATTCCCCCCCCCCCCCCCCCCA-3′), that hybridized specifically to the poly(G) insertion. Sizes were calculated by comparison to end-labeled DNA markers depicted at *right*. All blots were quantitated and decay rates calculated with a Betascope. (*C*) The upper two curves represent the decay of full-length *MFA2* transcripts (●) Average of multiple experiments with wild-type *MFA2* mRNA; (■) average of multiple B5pGM experiments. Similar curves were derived from both B12pGM and B28pGM transcripts (data not shown). The lower curves represent the average length of the shortest poly(A) tail present at each time point as calculated in Decker and Parker (1993); (○) wild-type *MFA2* transcripts; (□) B5pGM transcripts.

UTR reduced translation to <1% of wild-type levels (data not shown). These observations are consistent with our prior results indicating that ribosome elongation in *cis* is not required for the normal decay rate, or pathway of degradation, for the *MFA2* mRNA (Beelman and Parker 1994).

A second important observation is that all three of these transcripts accumulated mRNA fragments not seen with the wild-type transcript (Fig. 1B). These fragments accumulated to the highest extent with the insertion of a poly(G) run (Fig. 1B), but they were also observed, albeit at greatly reduced levels, with transcripts that contain an insertion that forms a strong stem–loop, which might also be expected to be a structural block to exonucleases (data not shown). These fragments showed a precursor–product relationship with the full-length mRNA and only substantially increased after deadenylation (Fig. 1B). By comparing the amounts of these fragments and the full-length mRNA present at various times, we calculated that at least 80% of the *MFA2* tran-

scripts must be degraded by a pathway that leads to the production of this fragment. Because this estimate does not take into account any decay of the fragment, these data are consistent with the vast majority, if not all, of the *MFA2* mRNA being degraded in this manner. The small amount of these mRNA fragments present prior to the pulse of transcription (−10 lanes) is attributable to basal transcription from the *GAL1* upstream activating sequence (UAS) and to the fragments themselves being relatively stable as judged by their decay at later time points ($t_{1/2}$>45 min; see B28pGM panel in Fig. 1B).

On the basis of their size (Fig. 1B) and RNase protection experiments (Fig. 2), these fragments extend from the 5′ side of the poly(G) tract to the 3′ end of the mRNA. For example, the fragments derived from the B28pGM transcript are shortened at the 5′ end by ~41 nucleotides (Fig. 2A). These 41 nucleotides consist of the 28 nucleotides 5′ of the insertion site and 13 nucleotides from the inserted oligonucleotide sequence that precede the poly(G) tract (see Materials and methods). In addi-

Muhlrad et al.

Figure 2. RNase protection analysis of the 5' and 3' ends of the decay intermediate and the full-length *MFA2* transcripts. (*A*) RNase protection assay of the 5' end of B28pGM mRNA. Time points are relative to the time of transcriptional repression. (10) A time point (10 min) where the majority of the mRNA remaining in the B28pGM experiment has been converted into the shortened mRNA intermediate (see Fig. 1B). The RNA probe used extended from nucleotide 316 of the *MFA2* transcript to the *SphI* site 5' of the gene. As illustrated above, the RNA probe contains poly(C) insertions at B28 and B178 to allow the probe to hybridize to transcripts containing poly(G) in these positions (represented by cartoon). Because the mRNA in this experiment has a single poly(G) insertion at B28, the 3' end of the protected fragment is at nucleotide 179. The protected fragments of ~218 nucleotides (the full-length 5' end plus the poly(G) oligonucleotide insertion), ~177 nucleotides (the 5' end of the poly(G) trapped intermediate), and ~135 nucleotides (the 3' end fragment) are illustrated at *right*. RNase protection assays with RNAs containing the poly(G) insertion at other positions or multiple poly(G) insertions in the 5' UTR gave similar results (data not shown). The band at 151 nucleotides is from transcripts from the chromosomal *MFA2* gene. Sizes were calculated by comparison to size markers and a sequencing ladder (not shown). (*B*) RNase protection assays of the 3' ends of *MFA2* transcripts under conditions where mRNA decay fragments accumulated. The probe, depicted at the top, was an in vitro transcript that extended from the *BamHI* site 3' of the *MFA2* gene to the *BamHI* site within the *MFA2*-coding region (see Materials and methods). Samples are as follows: (WT) Wild-type *MFA2* transcript; (B28pGM) *MFA2* transcript with poly(G) inserted into the B28 *BglII* site. The sizes of the major and two minor 3' ends were 194, 189, and 172 nucleotides, respectively. The high level of the wild-type mRNA at 10 min reflects residual transcripts that have poly(A) tails of heterogeneous lengths. These transcripts are therefore more apparent when analyzed by this method, which is much more sensitive than Northern blots. Identical results were obtained in the analysis of the B5pGM and B12pGM constructs (data not shown).
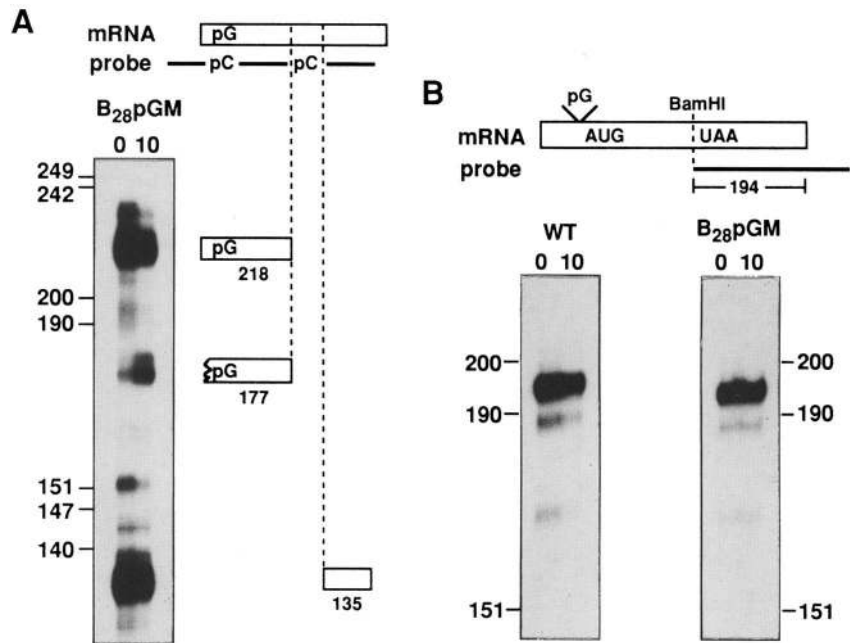
tion, the RNase protection assays indicate that these fragments have the same 3' ends as the full-length transcript (Fig. 2B). Moreover, because these fragments are shortened slightly by cleavage with RNase H and oligo(dT) (Fig. 1B, cf. 30 and 30dT lanes), they have oligo(A) tails, consistent with our previous results indicating that the decay step following deadenylation does not require the complete removal of the poly(A) tail (Decker and Parker 1993).

Similar results are obtained from the examination of *MFA2* transcripts that contain multiple insertions of the poly(G) sequence. The decay of a transcript that contains two tandem poly(G) insertions within the 5' UTR and a single poly(G) tract within the 3' UTR is shown in Fig. 3B. Although the overexposure of the autoradiographs to detect decay fragments obscures the deadenylation kinetics, the deadenylation rate was unaffected by the poly(G) insertions (data not shown). As would be expected if the poly(G) tracts are serving as a structural block to 5' → 3' digestion originating at the 5' end, the majority of the fragments have a 5' end at the most 5' poly(G) run within the 5' UTR. In addition, the level of the mRNA fragment stabilized by the poly(G) insertion in the 3' UTR is reduced relative to an mRNA that contains the same 3' poly(G) insertion but no secondary structure block in the 5' UTR (Fig. 3A). It should be

noted that because some fragments with a 5' end at a downstream poly(G) run are produced, the block to 5' → 3' exonucleolytic digestion is either not absolute or there are internal cleavage events that occur less frequently than the 5' cleavage event. However, the observation that the majority of the fragments contain a 5' end at the most 5' poly(G) tract provides strong evidence that the poly(G) tracts are acting as structural barriers to 5' → 3' degradation. Therefore, the results from both transcripts with single or multiple poly(G) insertions suggest that shortening of the poly(A) tail to an oligo(A) length allows one or more cleavage events near the 5' end of *MFA2* mRNA.

Analysis of MFA2 *mRNA degradation in* xrn1Δ *strains*

The analysis of *MFA2* transcripts with poly(G) inserted in the 5' UTR suggested that deadenylation leads to a nucleolytic cleavage event near the 5' terminus. However, because the poly(G) insertions do alter translation of the *MFA2* transcript, we desired to confirm this result by analyzing degradation of the *MFA2* transcript under conditions where 5' → 3' exonucleolytic degradation was blocked by a method that does not require the insertion of poly(G) tracts into the 5' UTR. A likely candidate for the enzyme responsible for the 5' → 3' degra-
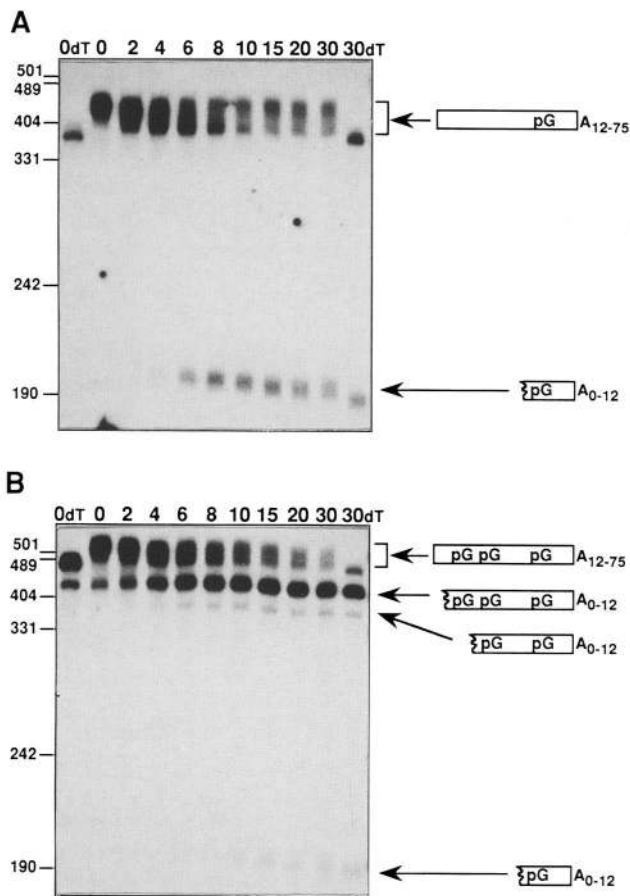
Figure 3. Analysis of the decay of *MFA2* transcripts with multiple poly(G) insertions. Decay of *MFA2* transcripts with a single poly(G) insertion in the *Bgl*II site at nucleotide 178 (*A*) or with multiple poly(G) insertions (*B*) analyzed by a transcriptional pulse–chase. In *B* the transcript has two insertions of the poly(G) tract in B28 and a single poly(G) tract in B178. To allow direct comparisons of the relative levels of different decay fragments, the blots were probed with the oligonucleotide oRP140 (5'-ATATTGATTAGATCAGGAATTCC-3'), which is complementary to the 3' UTR–poly(G) junction and thus will hybridize equally to all mRNA species examined. Cartoons (*right*) represent the mRNA species in each band.

that contains the poly(G) tract in its 3' UTR. This insertion of poly(G) into the 3' UTR does not affect the decay of the MFA2 transcript and leads to the accumulation in wild-type cells of a decay product corresponding to the 3' portion of the mRNA (Decker and Parker 1993). By comparing several experiments, there was no significant change in the deadenylation rate of the *MFA2* mRNA. However, the deadenylated form of the *MFA2* transcript was more stable in *xrn1Δ* cells (Fig. 4B) as compared with an otherwise isogenic yeast strain (Fig. 4A). This result indicated that the *XRN1* gene product is required for the rapid decay of the deadenylated *MFA2* mRNA. Consistent with this interpretation, the decay product stabi-
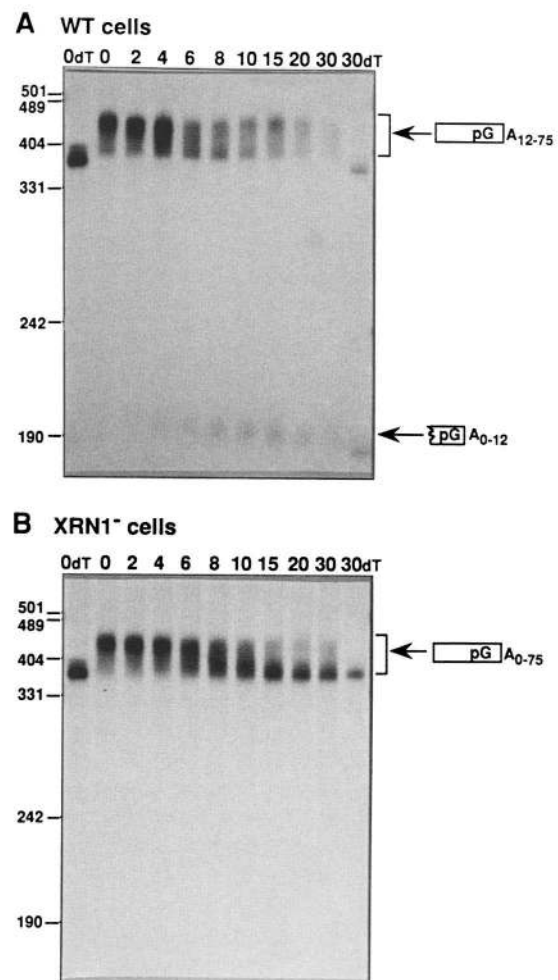
Figure 4. Deletion of the *XRN1* gene stabilizes the deadenylated species of the *MFA2* transcript. Transcriptional pulse–chase experiments of *MFA2* transcripts in isogenic wild-type (*A*; yRP582) and *xrn1Δ* mutant (*B*; yRP689) strains. The *MFA2* construct analyzed in both strains was expressed from a *LEU2* plasmid and contained a poly(G) insertion in its 3' UTR to allow detection of decay products and utilization of a poly(C) probe (see legend to Fig. 1) to specifically hybridize to this transcript and not endogenous *MFA2* transcripts (Decker and Parker 1993). Cartoons (*right*) depict the mRNA species in the Northern blot.

dation of mRNAs following deadenylation (Decker and Parker 1993) is a major cytoplasmic $5' \to 3'$ exonuclease in yeast encoded by the *XRN1* gene (Larimer and Stevens 1990; Hsu and Stevens 1993). Given this possibility, we examined the decay of the *MFA2* mRNA in a *xrn1Δ* strain by the transcriptional pulse–chase procedure. If deadenylation leads to cleavage near the 5' end and the *XRN1* gene product is responsible for the $5' \to 3'$ degradation of the body of the *MFA2* mRNA, then essentially full-length transcripts would be expected to persist following deadenylation.

The decay of the *MFA2* transcript in a yeast strain deleted for the *XRN1* gene is shown in Fig. 4. To evaluate the role of the *XRN1* gene product in the production of decay intermediates we utilized the *MFA2* transcript

lized by the poly(G) tract in the 3' UTR that is seen in wild-type cells (Fig. 4A) is lacking in xrn1Δ cells. It is interesting to note that in the xrn1Δ strain, the deadenylated form of the MFA2 transcript did eventually degrade. This observation suggests that there are additional, but slower, nucleases that can degrade this molecule (see Discussion).

The oligo(A) tail of the mRNA species that persisted in the xrn1Δ cells was observed to deadenylate to zero, or an extremely short length, at late time points (Fig. 4B, cf. 30 and 30dT lanes). Because shortening of the poly(A) tail to an oligo(A) length is sufficient to allow the mRNA to be a substrate for subsequent decay events (Decker and Parker 1993), this observation suggests that if the next step in mRNA decay is blocked, the continued shortening of the oligo(A) tail (terminal deadenylation) can continue. Terminal deadenylation is also observed on chimeric mRNAs containing sequences that slow decay steps after deadenylation (Decker and Parker 1993) and in the presence of cycloheximide, which also inhibits degradation after deadenylation (Beelman and Parker 1994).

Transcripts that accumulate in xrn1Δ strains are full length but decapped

Because the biochemical activity of the XRN1 gene product is a 5' → 3' exonuclease, the simplest explanation for the above results is that deadenylation triggers a cleavage event near the 5' end of the MFA2 mRNA and thereby exposes the transcript to 5' → 3' exonucleolytic degradation by the XRN1 gene product. However, because xrn1Δ strains have a multitude of phenotypes (Kim et al. 1990; Kipling et al. 1991; Dykstra et al. 1991; Tishkoff 1991), it was formally possible that the effect on MFA2 mRNA decay is an indirect consequence of the deletion of XRN1 and not due to the direct loss of a 5' → 3' exonuclease required for mRNA degradation. Because the XRN1 nuclease is blocked by the 5' cap structure of mRNAs (Stevens 1978), removal of the cap would be a required step if XRN1 is involved in the decay of MFA2 mRNA. Therefore, if the XRN1 gene product is directly involved in the degradation of the transcripts following removal of the cap structure, we would expect that the deadenylated transcripts that accumulated in the xrn1Δ strains would lack the 5' cap structure.

To determine whether or not the cap structure was being removed, we immunoprecipitated the MFA2 transcripts from early and late time points in the transcriptional pulse–chase with antisera directed against the cap structure (Munns et al. 1982). As shown in Figure 5, the MFA2 mRNAs from both wild-type and xrn1Δ cells are precipitable at the beginning of the chase period indicating that they are synthesized with a cap structure (lanes 2,5). At later times (15 min after transcriptional repression) the deadenylated transcripts that accumulate in xrn1Δ cells remain in the supernatant, thus indicating that they have lost the 5' cap structure (lane 9). These results indicate that the cap structure is removed by a nucleolytic event near the 5' end of MFA2 mRNA and
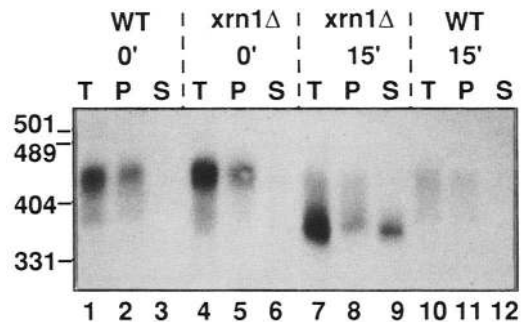

Figure 5. The deadenylated MFA2 transcripts that accumulate in xrn1Δ strains are uncapped. MFA2 mRNA containing a poly(G) insertion in its 3' UTR isolated from isogenic wild-type (WT) and xrn1Δ strains at the times indicated during transcriptional pulse–chase experiments was immunoprecipitated with antisera directed against the 5' cap structure (see Materials and methods). The MFA2 mRNA was detected by Northern analysis with the oligonucleotide oRP121 as a probe (see legend to Fig. 1). (T) The total amount of MFA2 mRNA in the samples before immunoprecipitation. Pellet and supernatant fractions are designated P and S, respectively. On average, ~25% of the mRNA was lost during the procedure, presumably during the repeated washes. As judged by control experiments precipitation required the presence of the anti-cap antibody and was specific to mRNAs containing a cap structure (data not shown).

suggest that the XRN1 nuclease is responsible for the 5' → 3' degradation of the body of the transcript (see Discussion). It should be noted that at 15 min following transcriptional repression any residual transcripts that persist with a poly(A) tail, either in wild-type, or xrn1Δ cells, remain precipitable (lanes 8,11). This observation provides additional evidence that deadenylation is required for the 5' cleavage event.

Because the deletion of the XRN1 gene allows the accumulation of decapped molecules, it should be possible to determine the site of the 5' cleavage event by mapping the 5' ends of these decapped mRNAs. To do this we examined by primer extension analysis the 5' ends of the transcripts from an early time point, when the mRNAs are still capped, and from a late time point, when the majority of the transcripts are decapped. As shown in Figure 6, the primer extension products in xrn1Δ cells are identical at both early and late times during the chase and reflect the normal 5' ends seen in wild-type cells. This result suggested that the 5' cleavage event that removes the cap structure did not change the length of the transcript. However, although the majority of the MFA2 transcripts in the 15-min time point examined are uncapped, some capped adenylated transcripts are present, which could contribute to primer extension products (see Fig. 5).

To clearly demonstrate that the uncapped MFA2 transcripts present at late time points in xrn1Δ cells were not shortened at the 5' end, we first separated the mRNA from the 15-min time point in xrn1Δ cells into capped and uncapped fractions utilizing the antisera directed against the cap structure. We then mapped the 5' ends of
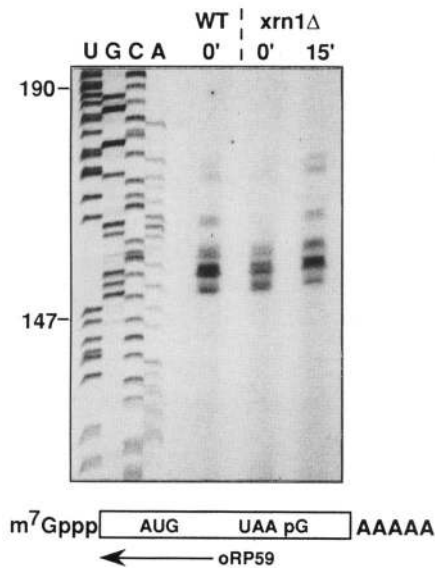
Figure 6. The *MFA2* mRNA species that accumulate in *xrn1Δ* strains are full length. Primer extension analysis of *MFA2* mRNA containing a poly(G) insertion in its 3' UTR isolated from isogenic wild-type (WT) and *xrn1Δ* strains at the times indicated during transcriptional pulse–chase experiments (see Materials and methods). The location of the primer oRP59 (5'-TTAAGCGATAACACAG-3') is illustrated below. The corresponding sequence of the sense strand of the *MFA2* gene is shown at *left*, as well as additional DNA size markers. The major extended product is 159 nucleotides long and corresponds to the major 5' end. Several less abundant products are also seen; their sizes are from *top* to *bottom*, 181, 178, 169, 163, 156, 153, 149, and 145 nucleotides.

the *MFA2* transcripts in each fraction. For comparison we also mapped the 5' ends of the *MFA2* transcripts from the 0-min time points from experiments in both wild-type and *xrn1Δ* cells. Because contaminants from the immunoprecipitation inhibit the primer extension analysis (data not shown), we analyzed the 5' ends in this experiment by an RNase protection assay that identifies essentially the same 5' ends as the primer extension assay. As shown in Figure 7, the 5' ends present in wild-type cells and *xrn1Δ* cells at time zero are identical. Moreover, the same 5' ends were present on capped and uncapped transcripts following immunoprecipitation of the 15-min time point from *xrn1Δ* cells (*xrn1Δ* 15-min P and S lanes). This observation indicated that the uncapped transcripts persisting in *xrn1Δ* cells have the same 5' end as the capped species and provided additional information verifying that the uncapped *MFA2* transcripts that persist in *xrn1Δ* cells are full length.

Discussion

Deadenylation leads to decapping of the MFA2 mRNA

Several lines of evidence indicate that deadenylation of the unstable *MFA2* transcript leads to nucleolytic cleavage at its 5' end. First, insertion of strong RNA secondary

structures into the 5' UTR led to the accumulation, after deadenylation, of mRNA fragments shortened only at their 5' end (Figs. 1, 2, and 3). Similarly, cells treated with cycloheximide, which stabilizes the oligoadenylated form of the *MFA2* mRNA, accumulate fragments of the *MFA2* transcript shortened at the 5' end (Beelman and Parker 1994). Finally, in strains deleted for the *XRN1* 5' → 3' exoribonuclease, *MFA2* transcripts accumulated following deadenylation (Fig. 4). The oligoadenylated species of the *MFA2* mRNA that accumulated was full length but lacked the 5' cap structure (Figs. 5, 6, and 7). In each case, we interpret these mRNA fragments to be intermediates in the decay pathway that are normally not observed as a result of being extremely short-lived. However, it is a formal possibility that the intermediates observed in each case represent aberrant products that form because the normal mRNA decay pathway is blocked. Two observations suggest that the observed intermediates reflect the normal decay mechanism. First, in each of the three cases described above, mRNA fragments accumulated following deadenylation that are altered at the 5' end, consistent with the same decay pathway operating under each condition. Second, the poly(G) insertions in the 5' end do not alter the requirement for deadenylation before decay, the rate of decay, or the rate of deadenylation, indicating that the poly(G) insertions
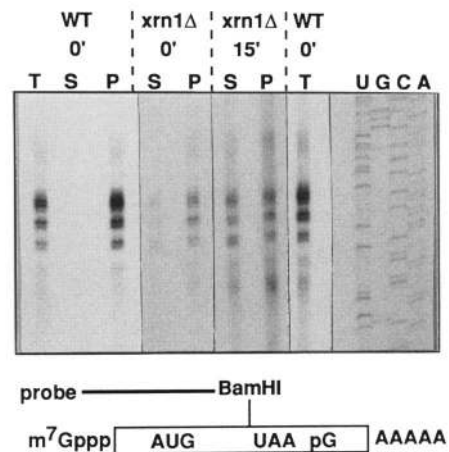

Figure 7. The uncapped *MFA2* mRNAs that accumulates in *xrn1Δ* cells are full length. Shown is RNase protection analysis of the 5' ends of *MFA2* transcripts containing poly(G) insertions in their 3' ends isolated from wild-type and *xrn1Δ* strains at the indicated time points of transcriptional pulse experiments. Lanes are labeled as follows: (WT) Wild-type cells; (*xrn1Δ*) *xrn1Δ* cells; with the time point of the sample analyzed given in minutes. Individual lanes are labeled as T (total RNA); P (pelleted RNA after immunoprecipitation with anti-cap antibodies); and S (RNA remaining in the supernatant after immunoprecipitation). The probe for this experiment extends from the end of the *Bam*HI site at nucleotide 140 of the mRNA to the *Sph*I site 5' of the gene. For size markers the sequence of the *MFA2* gene with oligonucleotide oRP59 (see legend to Fig. 6) is shown at *right*. Note that this sequence does not directly correlate between the position and the size of the protected fragments.

do not change the mechanism of decay. On the basis of these observations, we conclude that deadenylation of the *MFA2* transcript leads to cleavage at the 5' end of the transcript, removing the cap structure, and thereby exposing the transcript to 5' → 3' exonucleolytic degradation. In combination with our prior results describing discrete phases of deadenylation that are required for decay of the *MFA2* transcript (Muhlrad and Parker 1992; Decker and Parker 1993), these observations define the major decay pathway for the *MFA2* mRNA (Fig. 8).

The decay pathway that we have described requires a nucleolytic cleavage near the 5' end. Three observations suggest that the position of this cleavage on the *MFA2* transcript is 5' of the first encoded nucleotide. First, within the resolution of acrylamide Northern analysis, the uncapped transcripts present in *xrn1Δ* cells are full length (Fig. 5). Second, primer extension analysis of the *MFA2* transcripts present at 15 min after transcriptional repression in *xrn1Δ* cells produced cDNAs that extend to the same 5' end as the initial capped transcript (Fig. 6).
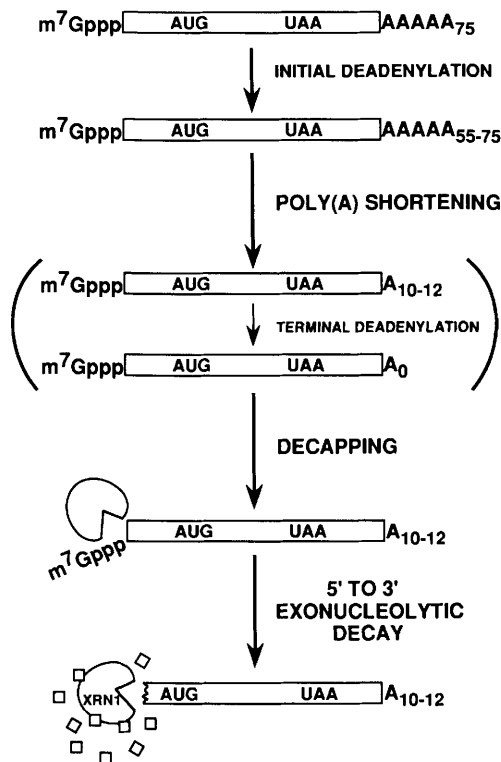

Figure 8. Decay pathway for the *MFA2* mRNA. In this pathway, shortening of the poly(A) tail leads to decapping of the transcript followed by 5' → 3' exonucleolytic degradation of the transcript by the *XRN1* exonuclease. The factors responsible for poly(A) shortening and decapping have not yet been defined. The poly(A) tail is shortened in several phases. An initial slow phase (initial deadenylation) precedes a more rapid phase [poly(A) shortening], during which the poly(A) tail is shortened to an oligo(A) length. Complete removal of the poly(A) tail (terminal deadenylation) is not required for decapping and is therefore placed in parentheses. The illustrated size of the poly(A) tails at the different steps is approximate.

Because the majority of the transcripts at this late time point are uncapped (Fig. 5), any cleavages near the 5' end should have been detected as shorter cDNAs. Finally, RNase protection analysis of the 5' ends of transcripts separated by immunoprecipitation into capped and uncapped populations indicated that the capped and uncapped *MFA2* transcripts gave rise to identical protected fragments (Fig. 7). Although we cannot define the precise bond that is cleaved, we interpret these observations to indicate that the decapping reaction does not remove any nucleotides from the *MFA2* transcript and therefore must be 5' of the first encoded nucleotide. It is interesting to note that in the steady-state analysis of the rp51A mRNA in *xrn1Δ* cells, a transcript was identified that was missing two bases at the 5' end (Hsu and Stevens 1993). Because it is not known whether the rp51A mRNA requires deadenylation before decay, this shortened transcript could be attributable either to a deadenylation-independent cleavage or to a deadenylation-stimulated decapping reaction. Moreover, an initial cleavage event at the 5' end could be followed by the subsequent trimming of two additional nucleotides on the rp51A mRNA. However, this observation raises the possibility that the site of cleavage for deadenylation-stimulated decapping may vary between mRNAs (see below). Future experiments will be needed to resolve this issue.

How would deadenylation stimulate decapping of the mRNA? One possibility is that decapping is an indirect result of deadenylation. For example, because the poly(A) tail of mRNAs can associate with the cytoskeleton (Lenk et al. 1977; Taneja et al. 1992), poly(A) tail shortening could alter the subcellular localization of an mRNA and thereby expose the mRNA to a decapping enzyme. An alternative model is that the poly(A) tail and factors associated with it, such as poly(A)-binding protein (PAB), might inhibit the decapping reaction directly by forming, or stabilizing, an mRNP structure that involves the 5' and 3' termini of the mRNA. Such an mRNP structure has been suggested previously on the basis of the stimulation of translational initiation by poly(A) tails (e.g., Doel and Carey 1976; Galili et al. 1988; Sachs and Davis 1989; Munroe and Jacobson 1990) and on the presence of circular polysome structures in electron micrographs (e.g., Christensen et al. 1987). Such an interaction is also supported by observations suggesting that base-pairing between the 5' and 3' UTR sequences can occur in vivo (Van den Heuvel 1990; Laso et al. 1993). One simple model is that the same mRNP structure that stimulates translational initiation could inhibit decapping, perhaps by efficiently recruiting translation initiation factors to the 5' UTR and cap structure.

Control of mRNA decay rate

Our results suggest that the instability of the *MFA2* transcript is attributable to the rapid execution of two key steps in its decay pathway, deadenylation and decapping. This conclusion suggests that features of individual

mRNAs that modulate the rates of deadenylation and decapping will contribute to differences in mRNA decay rates. Several sequences have been identified that promote degradation by accelerating deadenylation, including sequences within the *MFA2* 3' UTR and the AU-rich instability element found in the 3' UTR of c-*fos*, c-*myc*, and other unstable mammalian mRNAs (Wilson and Triesman 1988; Shyu et al. 1991; Muhlrad and Parker 1992). Mutations within the *MFA2* 3' UTR can also slow the decay of the deadenylated transcript (Muhlrad and Parker 1992), suggesting that these sequences may be required for rapid decapping. The AU-rich instability elements in mammalian cells have also been shown to promote mRNA degradation steps that occur after deadenylation (Shyu et al. 1991). Although it remains to be established whether any mammalian mRNAs are decapped following deadenylation, by analogy to the *MFA2* transcript, the AU-rich instability element may also function to promote rapid decapping.

How might the *MFA2* 3' UTR function to accelerate decapping? One model is suggested by the possibility that an interaction between the 5' and 3' termini exists that functions to stimulate both translation initiation and prevent decapping (see above). Sequences in the 3' UTR may disrupt, or alter, this $5' \rightarrow 3'$ interaction and thus affect the rates of translation and decapping. Given this possibility, it is striking to note that the AU-rich instability elements from mammalian cells have been shown to decrease translational efficiency independent of their effect on poly(A) tail shortening (Kruys et al. 1987, 1989; Grafi et al. 1993).

Alternative mRNA decay pathways

Our results suggest that additional mRNA decay mechanisms can operate on the *MFA2* transcript, although these mechanisms act more slowly than the primary decay pathway. This conclusion is supported by the observation that the decay intermediates that accumulated when $5' \rightarrow 3'$ exonuclease digestion was inhibited eventually were degraded. The decapped species that accumulated in *xrn1*Δ cells was less stable ($t_{1/2} = \sim 20$ min; Fig. 4) than the fragments trapped by poly(G) ($t_{1/2} > 45$ min, Fig. 1). One explanation for this observation is that there is an additional $5' \rightarrow 3'$ exonuclease, which is also effectively blocked by the poly(G) secondary structure, that can substitute for the *XRN1* gene product. A second major $5' \rightarrow 3'$ exonuclease in yeast is encoded by the *RAT1* gene (Kenna et al. 1993). Although by several criteria this nuclease appears to be predominantly a nuclear protein (Amberg et al. 1992; Kenna et al. 1993), it is possible that this enzyme may function in the cytoplasm to degrade decapped mRNAs. Consistent with this possibility, like the *XRN1* nuclease, the *RAT1* nuclease is also blocked by a poly(G) tract in vitro (A. Stevens, pers. comm.). Whether or not the *MFA2* mRNA can be degraded at a slow rate by $3' \rightarrow 5'$ exonucleases or internal endonuclease cleavage remains to be established.

Gene products involved in a mRNA degradation pathway

The pathway of mRNA decay that we have described requires several specific gene products. These factors include a poly(A) nuclease, a decapping enzyme, and a $5' \rightarrow 3'$ exonuclease as well as gene products that could modulate the rate of these reactions in a mRNA-specific manner. A candidate for the poly(A) nuclease in yeast, PAN1, has been identified and cloned on the basis of its requirement for PAB in vitro (Sachs and Deardorff 1992). It is interesting that mutations in this gene appear to primarily affect translation but have limited effects on poly(A) tail distributions. Therefore, it remains to be established whether PAN1 is the nuclease responsible for poly(A) shortening in vivo. The enzyme that performs the decapping reaction may correspond to a biochemically defined activity in yeast that preferentially decaps long mRNA substrates (Stevens 1988). The observation that uncapped, deadenylated transcripts accumulated in *xrn1*Δ strains is biochemically consistent with the interpretation that the *XRN1* gene product is a $5' \rightarrow 3'$ nuclease involved in mRNA turnover (Figs. 4 and 5; Hsu and Stevens 1993). The involvement of this exonuclease in mRNA degradation could explain why mutations in this gene have been reported to affect a wide range of biological processes (Kim et al. 1990; Dykstra et al. 1991; Kipling et al. 1991; Tishkoff 1991).

Generality of deadenylation and decapping as a mechanism of mRNA decay

Additional observations lead us to speculate that deadenylation leads to decapping of other yeast mRNAs and perhaps transcripts in other eukaryotes. Several mRNAs examined in *xrn1*Δ strains accumulate, under steady-state conditions, as poly(A)-deficient molecules lacking the 5' cap structure (Hsu and Stevens 1993). This observation is consistent with deadenylation triggering decapping of these transcripts. In addition, mRNAs lacking the cap structure are rapidly degraded in many eukaryotic cells (Furuichi et al. 1977; Green et al. 1983; Drummond et al. 1985; Gallie 1991). Moreover, enzymes capable of catalyzing the removal of the cap structure and subsequent $5' \rightarrow 3'$ degradation of the transcript have been described in mammalian cells (Nuss et al. 1975; Nuss and Furuichi 1977; Stevens and Maupin 1987; Murthy et al. 1991; Coutts and Brawerman 1993; Coutts et al. 1993). However, although these observations are suggestive, there is currently no direct evidence to indicate that mammalian transcripts are decapped following deadenylation.

It should be noted that because the poly(A) tail and the cap structure are common to essentially all eukaryotic mRNAs, a mechanism of degradation involving these features could function on many transcripts. The presence of sequence elements within mRNAs that affect the rate of poly(A) shortening and/or decapping, along with mRNA-specific turnover pathways, such as sequence-specific endonucleolytic cleavage (Binder et al.

Muhlrad et al.

1989; Stoeckle and Hanafusa 1992; Brown et al. 1993), could produce the entire diversity of mRNA decay rates observed in eukaryotic cells.

Materials and methods

Yeast strains and medium

Two yeast strains were used in this analysis: yRP582 (*MATa*, *rpb1-1*, *ura3-52*, *leu2*) and an isogenic *xrn1Δ* deletion strain yRP689 (*MATa*, *rpb1-1*, *ura3-52 leu2*, *xrn1Δ::URA3*) created by disruption with the construct dst2-1 (dst2Δ::URA3) (Dykstra et al. 1991). The *xrn1Δ* deletion was confirmed by Southern analysis (data not shown). Yeast stains were transformed as described in Schiestl and Gietz (1989). Yeast medium was prepared by standard techniques, and plasmids were maintained by growth in the corresponding selective media.

mRNA analysis

Analysis of mRNAs by the transcriptional pulse–chase technique was performed essentially as described in Decker and Parker (1993), with the following minor modifications. Medium containing raffinose was adjusted to pH 6.5 with NH_4OH to reduce acid hydrolysis of raffinose into galactose. In addition, the harvested cells were resuspended directly in media containing galactose.

mRNA was isolated as described in Caponigro et al. (1993). RNase H cleavage reactions and polyacrylamide Northern gels were performed as described in Muhlrad and Parker (1992). RNase protections were performed with RNase One (Promega) as per manufacturer's instructions by use of antisense transcripts produced by T7 transcription from pRP490 or pRP515 to analyze the 5' ends and from pRP507 to analyze the 3' ends of the mRNAs (see below for plasmid descriptions).

Immunoprecipitations were performed essentially as described in Yang et al. (1992), with polyclonal anti-m^{7}G antibodies produced by T.W. Munns (Munns et al. 1982) and graciously provided by E. Lund and J. Dahlberg. Anti-m^{7}G antibodies were prebound at 4°C to protein A–Sepharose CL-4B(Sigma) in IPPH (500 mM NaCl, 10 mM Tris-Cl at pH 7.5, 0.1% NP-40). The beads were washed five times with IPPL (150 mM NaCl, 10 mM Tris-Cl at pH 7.5, 1 mM EDTA, 0.05% NP-40) and subsequently incubated at 4°C with total mRNA in IPPL containing 1 mM DTT and 0.1 U/µl of RNasin (Promega) for 16 hr. The supernatant was recovered and incubated a second time with antibody prebound to beads. The pellets were washed three times with IPPL and combined. Bound and unbound mRNA was then isolated from the pellet and supernatant fractions and analyzed on polyacrylamide Northern gels.

Plasmid constructions

Plasmids expressing *MFA2* constructs containing 5' UTR poly(G) insertions were constructed in the following steps. First, a plasmid, pRP493, was constructed (see Beelman and Parker 1994), which contains the *MFA2* gene under the control of the *GAL1* UAS (pRP410; Decker and Parker 1993) in the vector pRP270 (Muhlrad and Parker 1992). The plasmid pRP493 contains the *TRP1* gene, the M13 origin of replication, CEN, and an autonomously replicating sequence (ARS) (with the *BglII* site in the ARS destroyed by filling in with Klenow fragment and religating). Second, site-directed mutagenesis was performed on pRP493 to introduce three *BglII* sites into the 5' UTR of *MFA2*. The sites constructed and oligonucleotides used were (1) B5 (oRP133: 5'-GATGATAGATCTCTGGTGTC-3') yielding plas-

mid pRP526; (2) B12 (oRP134: 5'-GTATGAAGATCTTAGC-TCGCTGG-3') yielding plasmid pRP527; and (3) B28 (oRP151: 5'-AGGTTGGTAGATCTTGTTGTAT-3') yielding plasmid pRP455.

Poly(G) tracts were inserted into the above plasmids by cutting with *BglII* and inserting the annealed oligonucleotides oRP126 (5'-GATCTAGGAATTTGGGGGGGGGGGGGGGGGG-GGAATTCCT-3') and oRP127 (5'-GATCAGGAATTCCCC-CCCCCCCCCCCCCCAAATTCCTA-3'). The resulting plasmids are referred to as B5pGM (pRP586), B12pGM (pRP595), and B28pGM (pRP596).

To allow selection of these plasmids in the *ura3* yeast strain yRP582, the *SacI–HindIII* fragment, containing the *MFA2* gene, was used to replace the corresponding fragment in pRP410, a *URA3* vector (Decker and Parker 1993). The resulting plasmids were pRP537 (B5M), pRP538 (B12M), pRP596 (B28M), pRP577 (B5pGM), pRP578 (B12pGM), and pRP505 (B28pGM).

The plasmid containing a tandem poly(G) insertion at B28 was created by cutting B28pGM (pRP505) with *BglII*, which cuts immediately 5' of the poly(G) tract, inserting the oligonucleotides oRP126/oRP127, to create a plasmid with two consecutive poly(G) tracts inserted at B28. This plasmid is referred to as B28pGpGM (pRP540). To introduce an additional poly(G) tract in the 3' UTR, a 400-base *BamHI* fragment from pRP485 (Decker and Parker 1993) containing a poly(G) tract in the 3' UTR was used to replace the corresponding *BamHI* fragment of pRP540 yielding the plasmid pRP575 (B28pGpGMpG).

To allow plasmid selection in the *xrn1Δ* strain, yRP689, the *MFA2* gene with a poly(G) tract in its 3' UTR, was moved to a *LEU2* plasmid pRS315 (Sikorski and Heiter 1989). This was done by adding *XhoI* linkers (no. 50001 from Promega) to the *HindIII* site at the 3' end of the *MFA2* gene and to the *EcoRI* site 5' of the *GAL1* UAS and inserting this *XhoI* fragment into the *XhoI* site of pRS315. This plasmid is referred to as pRP590.

The plasmid used to perform RNase protections on the 5' ends of the B28pGM mRNA, pRP490, contained the *MFA2* sequence with poly(G) inserted in both B28 and B178 and extended from an *SphI* site (blunt ended), ~300 bases 5' of the mRNA start, to a *BglII* site at base 316 of the *MFA2* mRNA subcloned into the vector pGEM-3Z at the *KpnI* (blunt ended) and *BamHI* sites. This plasmid was linearized with *HindIII* and transcribed with T7 RNA polymerase (Promega) to produce the antisense strand.

The plasmid used for RNase protections of the 5' end of *MFA2* transcripts after immunoprecipitation with anti-cap antibodies was produced by T7 transcription of a *HindIII* linearized plasmid, pRP515, which contains the 380-base *SphI–BamHI* fragment of wild-type *MFA2* from pRP270 (Muhlrad and Parker 1992) inserted into pGEM-3Z.

The plasmid used in the RNase protections to analyze the 3' ends of the *MFA2* transcripts, pRP507, was created by subcloning the 400-nucleotide *BamHI* fragment containing the 3' end of the wild-type *MFA2* gene into the *BamHI* site of the vector pGEM-4Z, in the orientation that linearization with *EcoRI* and transcription with T7 RNA polymerase (Promega) produced the antisense strand.

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Deadenylation of the unstable mRNA encoded by the yeast MFA2 gene leads to decapping followed by 5'-->3' digestion of the transcript.

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