Deadenylation is the rate-limiting step of mRNA decay, yet little is known about the mechanism regulating this process. In yeast, deadenylation is mainly mediated by the Pop2-Ccr4 complex. We tested whether the selective recruitment of this deadenylation to target mRNAs was sufficient to stimulate their decay in vivo. For this purpose, the Pop2 factor was fused to a U1A RNA binding domain while U1A binding sites were inserted in untranslated regions of a reporter transcript. Analysis of the reporter fate in strains expressing the Pop2-U1A-RBD fusion demonstrated a specific activation of target mRNA decay. Increased mRNA degradation involved accumulation of deadenylated mRNAs that was not detected when the control factors Dcp2 or Pub1 were tethered to the same transcript. The rapid target mRNA degradation was also accompanied by the appearance of new decay intermediates generated by the 3'–5' trimming of the corresponding 3'-untranslated region. Interestingly, this process was not mediated by the exosome but may result from the activity of the Pop2-Ccr4 deadenylase itself. These results indicate that selective recruitment of the Pop2-Ccr4 deadenylase is sufficient to activate mRNA decay, even though this process can also be stimulated by additional mechanisms. Furthermore, deadenylase recruitment affects the downstream path of mRNA decay.

Regulation of gene expression is a fundamental process occurring in all organisms. Since messenger RNAs were hypothesized to carry the information contained in the genome to protein-synthesizing ribosomes, it has become clear that gene regulation could be controlled at many steps, including transcription and mRNA decay (1). Although the process of transcription has been analyzed for a long time in eukaryotes, transcription and mRNA decay (10, 11). Surprisingly, both the Pop2 and Ccr4 subunits of this large assembly are endowed with catalytic nuclease activities (7, 12–14). The major catalytic activity of mRNA turnover in human cells occurs by 3'–5' direction. It involves deadenylation of the substrate mRNA that induces cap cleavage before 5'–3' exonucleolytic degradation of the mRNA body. A minor degradation pathway also involves mRNA deadenylation. However, this step is then followed by 3'–5' degradation of the mRNA body. These pathways appear to be conserved from yeast to human cells. In the latter case, cytoplasmic degradation of specific mRNAs has also been shown to be initiated by endonucleolytic cleavage (4). It has been suggested that the majority of mRNA turnover in human cells occurs by 3'–5' degradation (5).

Deadenylation is a common step in the degradation of normal mRNAs by the 5'–3'- and 3'–5' pathways. This process has been shown to be rate-limiting (6). In yeast, deadenylation is mostly mediated by the Pop2-Ccr4 complex (7, 8), which degrades poly(A) tails of substrate mRNAs with kinetics varying for each target substrate. Deadenylation proceeds until 10–15 A residues are left on the target mRNA. In many cases, this shortening induces, by an unknown process, the cleavage of the mRNA cap by the Dcp2 factor (3), whereas in other cases deadenylation is followed by the exonucleolytic 3'–5' decay of the mRNA body that is mediated by a multienzyme complex called the exosome (9).

The major yeast deadenylase was identified as the Pop2-Ccr4 complex (7, 8), which was originally suggested to repress transcription (10, 11). Surprisingly, both the Pop2 and Ccr4 subunits of this large assembly are endowed with catalytic nuclease activities (7, 12–14). The major catalytic activity in vivo can be attributed to Ccr4 because point mutations in its active site destroys catalytic activity (7, 12). Additional deadenylases have been identified, including PARN in vertebrates (19), the Pan2-Pan3 complex in yeast and human (20, 21), and Nocturnin in Xenopus laevis (22). PARN was suggested to be the major deadenylase in vertebrate cells. However, this idea was challenged as it appears to be conserved only in this evolutionary group, and functional analysis indicated a major role for Pan2.
and Pop2-Ccr4 in mRNA deadenylation (21). In contrast, genetic analyses demonstrated that the Pop2-Ccr4 complex represents the major deadenylase in yeast and Drosophila (7, 12, 23), whereas deletions of Pan2 or Pan3 were found to have few consequences on mRNA turnover in yeast (24).

Because deadenylation is the rate-limiting step for mRNA decay, it constitutes a major regulatory step for mRNA stability (6). However, little is known about its regulation. The Pop2-Ccr4 complex has been shown to be associated with several other factors, Notl-5, Caf130, and Caf40, that have been shown to contribute to its regulation through unknown mechanisms (10–12). These observations do not explain, however, the differences observed in the speed of deadenylation between mRNAs originating from distinct genes. Different hypotheses can be made to explain these variations. An extreme hypothesis could be that the deadenylase is constitutively active. If this is the case, different mRNA half-lives would result from a selective recruitment of the active complex to substrate mRNA. Such recruitment could be mediated via a bridging factor recognizing specific signals on the messenger. Alternatively, an inactive deadenylase complex could be turned on locally, e.g. by a post-translational modification. If the first hypothesis is correct, tethering the deadenylase to a stable messenger should be sufficient to promote its rapid deadenylation, as this artificial situation would bypass the recruitment step. In contrast, if local activation controls deadenylase activity, tethering of the deadenylase should not increase deadenylation as it should still require triggering by a local activator.

In this study, we developed a general tool based on the human U1A binding domain (25) to target protein factors to reporter mRNAs in yeast. When Pop2 was targeted to a stable PGK1 reporter mRNA, the resulting deadenylation activity was more processive and the half-life of the transcript was decreased 2-fold. Furthermore, we were able to detect a new decay intermediate induced by the nuclease activity of the tethered deadenylase. These results indicate that deadenylation can be regulated by recruitment of the deadenylase to target transcripts. In addition, these results show that the constructs that we prepared for the rapid fusion of the U1A cassette to target genes by genomic integration are functional. These constructs should prove powerful to study the function of yeast factors involved in mRNA decay or other RNA-dependent processes, particularly for genes without clear phenotypes.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—The Escherichia coli strains MH1 and MC1066 were used for cloning that was performed following standard procedures. All yeast strains used in this study originated from W303 or its derivative BMA64. The BY1170, BSY1172, and BSY1574 strains contain the sequence coding the U1A RNA binding domain fused downstream of the last encoded amino acids of POP2, DCP2, and PUB1, respectively. These were constructed by PCR-mediated gene tagging (26) using pBS2215 (see below) as template. The ccr4 disruption was obtained by insertion of the HIS3-MX6 cassette (27) in the BSY1171 diploid strain that is heterozygous for the Pop2-U1A-RBD fusion. After sporulation, a meiotic segregant, BSY1505, was selected. The BSY1487 strain harboring the dis3 thermo-sensitive mutation and the Pop2-U1A-RBD fusion was obtained by crossing the two cognate strains and dissection of the resulting diploid. Standard complete and synthetic media were used for growing E. coli and yeast strains.

**Plasmid Construction**—Plasmid pBS2215 was generated by inserting the sequence encoding the first 102 amino acids of the human protein U1A (corresponding to its RNA binding domain) and a HA4 tag from plasmid pG1hU1A- HA (28) between the SacI and HindIII sites of plasmid pBS1479 (29). Reporter plasmids were first generated by cloning a fragment of plasmid p602RP (30) encompassing the GAL1 UAS, PGK1 promoter, PGK1-coding sequence and its 3'-UTR, an oligo(G), and the polyadenylation site of PGK1 between the NotI and HindIII sites of pRS416 (31), generating pBS2284. The sequence of the human U1A stem-loop binding site was inserted either in the 5'-UTR (pBS2324) or in the 3'-UTR (pBS2322) by cloning PCR fragments, respectively, between the XbaI and HindIII sites or the BamHI and MfeI sites of pBS2284. The same strategy was used to insert the control sequences complementary to the U1A binding stem-loop, producing plasmids pBS2325 (5'-UTR) and pBS2323 (3'-UTR). Sequences of the 3'-UTR insertions are given in Fig. 1C. The 5'-UTR insertions are 5'-taaccttttaaggatccattgcaactccggatTTTCATAcTTGTaaaatat-3' for the binding site and 5'-taaccttttaaggatccattgcaactccggatTTTCATAcTTGTaaaatat-3' for the non-binding site (uppercase letters denote the inserted sequence and lowercase letters the flanking PGK1 sequence).

Insert sequences derived from PCR or oligonucleotide cloning were confirmed by sequencing. This revealed that the poly(G) sequences inserted in pBS2322 and pBS2323 contained 17 Gs, whereas 18 Gs were present in pBS2324 and pBS2325. The oligo(G) tracks were thus identical between matched plasmids. These oligo(G) tracks were sufficient to promote accumulation of mRNA decay intermediates by blocking the Xrn1 exonuclease, irrespective of the presence of 17 or 18 G residues (see “Results”).

**RNA Analysis**—To map the polyadenylation site of the PGK1 transcripts, total yeast RNA was first reverse transcribed using primer OBS 1320 (see supplemental Table S1 for oligonucleotide sequences) and AMV reverse transcriptase (Stratagene). cDNAs were then PCR amplified using two primer combinations, OBS 34 and OBS 1321 or OBS 34 and OBS 1322 for 30 cycles using Taq DNA polymerase. The products obtained were cloned using the TOPO cloning system from Invitrogen. Several clones were sequenced to identify polyadenylation sites.

Reporter plasmids were introduced into yeast strains by LiCl-mediated transformation (32). RNA chase experiments were performed as described previously (7). Briefly, yeast total RNA was extracted using hot acid phenol extraction. Northern blot analyses were carried out following either formaldehyde-agarose or polyacrylamide gel electrophoresis. The reporter PGK1 mRNA was detected using oligonucleotide probes complementary to different regions of the PGK1 reporters.

3 F. Lacroute, unpublished information.

4 The abbreviations used are: HA, hemagglutinin; UTR, untranslated region; RBD, RNA binding domain; BS, binding site.
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(OBS1220–1223, supplemental Table S1). Radioactive signals were collected and quantified using a phosphorimager (GE Healthcare). They were used to derive mRNA half-lives using the best fit for an exponential decay, and the latter averaged from at least two experiments.

Western Blots—Western blots using anti-HA antibodies (Covance) and horseradish peroxidase-coupled secondary antibodies were performed using a standard protocol and detected using chemiluminescence (33).

RESULTS

A System to Target a Deadenylase to a Reporter mRNA—As deadenylation is the rate-limiting step for mRNA decay, it is important to understand whether this process is controlled by recruiting a constitutively active deadenylase on target substrates or whether the enzyme is present in excess but only activated locally. Obviously, both modes of control are not entirely exclusive and each could contribute to some level of regulation. In an effort to discriminate between these two models, we decided to target the Pop2-Ccr4 complex on a reporter mRNA in the yeast *Saccharomyces cerevisiae*. We selected the stable PGK1 mRNA for this purpose, as it is deadenylated slowly and is highly stable (6). Hence, subtle increases in its deadenylation rate should sensitively lead to a reduced half-life. We selected the human U1A RNA binding domain (U1A-RBD) and its cognate RNA binding site (U1A-BS) to target Pop2-Ccr4 to PGK1 reporters, because this system has been extremely well characterized in previous structural studies (25) and because both constituents are relatively small and their affinity is high. Furthermore, the yeast U1A protein and its binding site in the yeast U1 snRNA are highly divergent from their human counterparts, thus excluding possibilities of undesired interference (34). Consistently, previous studies have revealed that ectopic expression of human U1A fusion is innocuous to yeast cells (28, 35). We constructed a plasmid encoding a cassette composed of a calmodulin binding peptide, a TEV cleavage site, the U1A-RBD, and the HA tag together with the TRP1 marker from *Kluyveromyces lactis* (Fig. 1A). This construct allows the rapid fusion of any yeast protein to the U1A-RBD using standard, PCR-based, genomic integration protocols (36).

We selected the Pop2, rather than Ccr4, subunit of the heterodimeric yeast deadenylase (7, 8) for C-terminal fusion with the targeting module because the nuclease domain of Ccr4 is located at its C terminus. As it has been proposed that Ccr4 is responsible for the major fraction of the deadenylase activity, addition of the U1A module to the Ccr4 may have interfered sterically with the function of this complex. Furthermore, previous experiments had demonstrated that fusion of tag sequences to the C terminus of Pop2 was not detrimental to cell growth (data not shown). We fused the U1A-RBD cassette downstream of, and in-frame with, the genomic POP2 locus in the haploid yeast strain W303a. This strategy maintains the expression of POP2 under the control of its natural promoter and ensures that all Pop2 molecules produced in these cells contain a U1A-RBD. As controls, we produced isogenic yeast strains with fusion of U1A-RBD to either Dcp2 or Pub1. Correct integration of the U1A-RBD at the targeted loci was confirmed by PCR; expression of the fusion proteins was demonstrated by Western blotting using an anti-HA antibody. Because the Pop2 function is required for normal cell growth and because the strain carrying the U1A-RBD-Pop2 fusion as the only source of Pop2 grows normally, we conclude that this fusion did not significantly alter its function. Similarly, the Dcp2 fusion strain did not display the very slow growth rate indicative of the inactivation of this gene, whereas for Pub1 it is known that it can be fused to a C-terminal tag without affecting its expression or correct localization (37).

We next needed to insert a U1A-BS in the PGK1 reporter. We chose to build constructs harboring this sequence in either
the 5′- or the 3′-UTR (Fig. 1B). To ensure the construction of a functional reporter, we first needed to map the exact polyadenylation site marking the extremity of the 3′-UTR, as this feature has only been determined indirectly in the past. Total yeast RNA from a W303 yeast strain derivative was used to generate cDNAs with an oligo(dt)-containing primer in a 3′ rapid amplification of cDNA ends experiment. Fragments overlapping the polyadenylation site of the PGK1 reporter were recovered by nested PCR and cloned. Sequences of several resulting clones revealed the location of the major polyadenylation site of the chromosomally encoded PGK1 mRNA (Fig. 1C). Interestingly, this site is more than 25 nucleotides upstream of the site reported in the literature from indirect RNA mapping experiments. With this information, we constructed clones containing one U1A-BS either in the 5′-UTR or in the 3′-UTR (Fig. 1C and “Experimental Procedures”). As controls, we inserted in the same location the antisense sequence unable to bind to the U1A-RBD (Fig. 1). Like the parent plasmids, all reporters also contain in their 3′-UTR an oligo(G) sequence that blocks the 5′-3′-Xrn1 exonuclease, allowing the detection of otherwise unstable mRNA decay intermediates. Furthermore, sequencing of rapid amplification of cDNA end clones derived from the reporter with the U1A-BS insertion confirmed that this modification did not change the site of polyadenylation.

**Insertion of a U1A Binding Site Does Not Significantly Alter Reporter mRNA Decay**—We first had to validate that insertion of the U1A-BS or the antisense control did not significantly alter the half-life and decay mode of the reporters. For this purpose a wild-type yeast strain was transformed with the reporter encoding plasmids carrying the U1A-BS, or the antisense control sequence, in their 3′-UTR. Transformants were grown in galactose-containing selective medium allowing the expression of the reporter before transfer to glucose-containing medium that shuts off reporter transcription allowing analysis of reporter mRNA stability. Total yeast RNA was extracted at different time points along this chase experiment, fractionated on agarose gels, and analyzed by Northern blot using an oligo(C) probe specific for the reporters. The results indicated that both transcripts follow essentially the same degradation pathway with similar kinetics (Fig. 2). Indeed, the larger band detected at time of transcriptional repression corresponds to the full-length mRNA, while a shorter intermediate characteristic of the 5′-3′ decay pathway was also detectable, the former species disappearing more rapidly than the latter one. Determination of the two mRNA half-lives, 24 ± 1.3 min, and 31 ± 0.5 min, respectively, indicated that both are stable species with half-lives within the range of values reported for the original p602RP construct (8, 30). Similar results were obtained for reporters containing insertions in the 5′-UTR (data not shown).

**Targeting the Pop2-Ccr4 Deadenylase to a mRNA 3′-UTR Speeds Its Decay**—The reporter plasmid harboring a U1A-BS in its 3′-UTR, and the antisense control, were introduced into the yeast strain producing the Pop2-U1A-RBD fusion (BSY1170). The decay of the encoded mRNA was assayed in a transcriptional chase experiment (Fig. 3). The first obvious result was that the mRNA amount was greatly reduced in the cells targeting Pop2 to the reporter RNA (4 ± 0.75-fold at the 0 time point). A similar reduction was seen for the corresponding decay intermediate. Second, the half-life of the targeted reporter mRNA in the Pop2-U1A-RBD strain (11.4 ± 1.1 min) was decreased ~2-fold compared either with the same reporter in a wild-type strain (t_1/2 = 24 ± 2.9 min, Fig. 2) or to the untargeted construct in the strain expressing the Pop2-U1A-RBD fusion (t_1/2 = 20.9 ± 1.3 min, Fig. 3). Third, a new RNA species slightly shorter than the full-length mRNA, possibly corresponding to a deadenylated species, was detected (Fig. 3, asterisk). These effects resulted specifically from the targeting of the deadenylase to the reporter mRNA 3′-UTR because (i) they were not observed in cells not expressing the deadenylase-U1A-RBD fusion, (ii) they were not observed with a control reporter unable to interact with the deadenylase, and (iii) they were not observed when the Pub1- or Dcp2-U1A-RBD fusions, which would simply bind but have no deadenylase activity, were targeted to the same reporter (Fig. 4 and data not shown). Interestingly, targeting of Pop2 to the 5′-UTR (which is also close to the poly(A) tail given the elf4G-Pab1 interaction) (38) also

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**mRNA Decay Induced by Targeted Pop2**

| Strain | Wild Type | Wild Type |
|--------|-----------|-----------|
| Reporter | Sense (pBS2322) | Antisense (pBS2323) |
| Time in glucose | | |
| min | | |
| 0 | 5 | 15 | 30 | 60 | 90 |
| 0 | 5 | 15 | 30 | 60 | 90 |

**FIGURE 2. Analysis of the decay of PGK1 reporters pBS2322 (U1A-BS), lanes 1–7, and pBS2323 (antisense), lanes 8–14, by transcriptional shut off in a wild-type strain.** Total RNA extracted at various time points after glucose-mediated repression of the GAL promoter was fractionated on an agarose gel, transferred to nylon membrane, and hybridized with an oligonucleotide complementary to the oligo(G) sequence (OBS1298, supplemental Table S1) to specifically detect the full-length mRNA and the decay intermediates. The lower part shows the hybridization of the membrane with a scr1 probe (OBS144, supplemental Table S1) that serves as an internal loading control. The time of sample analysis after transfer to glucose is indicated above each lane, while lanes 1 and 8 contain RNA extracted from cells grown in glucose (glc) overnight and thus represent the background signals originating from the reporter. Positions of migration of mRNA and intermediate are indicated.

**FIGURE 3. Analysis of the decay of PGK1 reporters pBS2322 (U1A-BS), lanes 1–7, and pBS2323 (antisense), lanes 8–14, by transcriptional shut off in the strain expressing the Pop2-U1A-RBD fusion.** The asterisk indicates an RNA species detected upon targeting of Pop2 that is slightly shorter (by ~60 nucleotides) than the full-length mRNA. The lower part shows the hybridization of the membrane with a scr1 probe that serves as an internal loading control. See Fig. 2 for other details.
mRNA Decay Induced by Targeted Pop2

A

| Strain         | Wild Type | Pop2-U1A-RBD | Dcp2-U1A-RBD |
|----------------|-----------|--------------|--------------|
| Reporter       | Sense (pBS2322) | Sense (pBS2322) | Sense (pBS2322) |
| M               | C 0 5 10 15 20 30 60 90 | C 0 5 10 15 20 30 60 90 |

Time in glucose (min)

mRNA Decay Induced by Targeted Pop2

B

mRNA

Intermediate

New Intermediate

FIGURE 4.

Analysis of the degradation of the PGK1 reporter pBS2322 (U1A-BS in its 3'-UTR) by transcriptional shut off in wild-type (lanes 2–10), in Pop2-U1A-RBD (lanes 11–19), and Dcp2-U1A-RBD (lanes 20–28) strains. Total RNA isolated as described for Fig. 3 was digested by RNase H after hybridization with an internal primer complementary to the PGK1 mRNA, fractionated by denaturing gel electrophoresis, transferred to nylon membrane, and hybridized with an oligonucleotide complementary to the poly(G). For each kinetic, lanes labeled with C correspond to the background signal observed when cells were grown in glucose overnight. Lanes labeled with D correspond to an RNA sample from time 0 treated by RNase H with an oligo(C) in addition to the PGK1-specific oligonucleotide, thus revealing the position of migration of deadenylated species. Lanes labeled with ND correspond to the time 0 sample before digestion by RNase H. In the Dcp2-U1A-RBD panel, the lane indicated with a star corresponds to the 5-min point of the Pop2-U1A-RBD kinetic that was duplicated to serve as a reference. Positions of migration of full-length mRNA, deadenylated full-length mRNA (FL A0), intermediate, and new intermediate are indicated. In addition, a DNA molecular weight marker was loaded in lane 1, and the sizes (in nucleotides) of the fragments are indicated on the left. B, structure of reporter mRNA and decay intermediates. The top panel displays the structure of the PGK1 reporter mRNA and its important features (not to scale), ORF (open reading frame), G (oligo(G) tract), U1A-BS, and poly(A) tail. The location of oligonucleotide OBS143 used to cleave total yeast RNA with RNase H is also indicated (line above the mRNA). The middle part shows the structure of the classical intermediate. The bottom part shows the structure of the new intermediate detected following targeting of Pop2 to the reporter mRNA.

decreased the stability of the reporter mRNA (data not shown). This result demonstrates further that the targeting effect does not result from a peculiar alteration of the reporter 3’-UTR.

It is noteworthy that targeting of Dcp2, which encodes the catalytic subunit of the yeast decapping enzyme, did not induce a faster decay of the mRNA reporter. Thus, not every mRNA decay factor tethered to an mRNA induces its degradation. This suggests that only some specific activities are able to engage attached mRNAs in the degradation pathway.

New mRNA Decay Intermediates Are Generated in the Presence of the Targeted Deadenylase—The Northern blot analysis of reporter mRNA and cognate decay intermediates fractionated on agarose gels is not precise enough to reveal the fine structure of the RNA decay intermediates. Therefore, to get higher resolution, we cleaved the reporter mRNA obtained in the chase experiment with RNase H and an internal primer (OBS143, Fig. 4B and supplemental Table S1). The resulting wild-type strain and rapid and processive following Pop2-Ccr4 targeting, even though other interpretations are possible. Comparison of the profile of the shorter mRNA decay intermediates revealed the presence of a new product of ~100 nucleotides in the targeted strain (Fig. 4A, lanes 12–19). Because this fragment was apparent before RNase H cleavage (lane 12) and because it hybridizes with the oligo(C) probe, its 5’ extremity can be mapped to the region downstream of the oligonucleotide used for cleavage but upstream of the oligo(G) sequence. Its apparent size and comparison with a deadenylated mRNA fragment suggest that its 3’-end occurs close to the inserted U1A-BS. However, it does not result from the binding of the U1A module to the mRNA, as it is absent in the Dcp2-U1A-RBD strain (lanes 21–28).

To characterize this intermediate further, we tested whether it was detected on Northern blot by a collection of oligonucleotides complementary to various locations downstream of the shorter 3’ fragments were then analyzed by high resolution polyacrylamide gel Northern blot (Fig. 4). Comparison of RNAs derived from the plasmid containing a U1A-BS in its 3’-UTR revealed several differences between a wild type or Dcp2-U1A-RBD and the deadenylase-targeted strain. First, consistent with the agarose gel analysis, these data indicated that the level of full-length polyadenylated species was lower in the targeted strain compared with the wild-type or Dcp2-U1A-RBD strains and that this species was more unstable in the former case (Fig. 4A, compare lanes 14–19 with lanes 5–10 or 23–28). Importantly, the increased decay rate of the reporter RNA induced by deadenylase targeting is associated with changes in the decay mode of the poly(A) tail. Inspection of the gel indicates that, whereas the size of the reporter mRNA poly(A) tail decreased homogeneously with time in a wild-type strain, decay in the targeted strain occurred with the time-dependent conversion of a population of mRNAs with full poly(A) tails to a population of mRNAs with very short (or no) poly(A) tails (e.g. lanes 14 and 15; see also supplemental Fig. S2 and the similar pattern observed with the di3 mutant strain shown in Fig. 5). Interestingly, little or no mRNAs with intermediate poly(A) tail size are detected. This result is consistent with a model in which deadenylation is slow and distributive in the...
mRNA Decay Induced by Targeted Pop2

Thus, targeting of the Pop2-Ccr4 deadenylase induces faster and more processive deadenylation of the reporter mRNA, resulting in its increased decay rate. Furthermore, this targeted activity changes somewhat the degradation pathway. In normal conditions, deadenylation removes the poly(A) tail up to the last 10–15 A residues before decapping occurs. However, when the deadenylation is tightly bound to its substrate, decapping still occurs but further 3′-5′ degradation appears to occur in addition. This degradation is apparently blocked (or slowed down) by the strong structure of the U1A-B5 bound by the U1A protein, leading to the appearance of the new mRNA decay intermediate.

Ccr4 Activity, but Not the Exosome, Is Required for Accumulation of the New mRNA Decay Intermediate—Formally, two different models can be proposed to explain the presence of the new mRNA decay intermediate: either the increased deadenylase rate generates a new entry point for another 3′-5′-exonuclease or the targeted deadenylase is directly responsible for this degradation. Indeed, because of the increased rate of poly(A) degradation, deadenylation may not stop when 10–15 A residues are left and targeted Pop2-Ccr4 may completely remove the reporter mRNA poly(A) tail, providing an entry point for other 3′-5′-nucleases.

The exosome would be the prime candidate for this role as it is currently the only 3′-5′-exonuclease that has been implicated in the decay of mRNA body. The alternative hypothesis implies that the deadenylase is itself able to degrade non-A residues beyond the poly(A) tail. Although deadenylases show a clear preference for A residues, they are known not to have an absolute substrate specificity (14, 16). To discriminate between these possibilities, we tested for the presence of the new decay intermediate in strains carrying the Pop2-U1A-RBD fusion and either a deletion of ccr4, the subunit responsible for the major part of the Pop2-Ccr4 deadenylase activity, or a thermosensitive mutant of the exosome (dis3). Interestingly, the new mRNA decay intermediate was readily detected in the mutated exosome strain grown at the non-permissive temperature (Fig. 5). In contrast, this intermediate was absent in the strain lacking the Ccr4 protein (Fig. 5). The absence of effect of exosome inactivation is not due to a residual activity, as the characteristic accumulation of 7 S rRNA processing intermediate resulting from exosome blockage was clearly detectable in the dis3 mutant (Fig. 5B, compare the main band intensity between the dis3 mutant and the control ccr4 mutant strain).

The targeting of Pop2 leads to the appearance of a shorter mRNA band (possibly extending from the cap to the U1A-BS) detected by blotting of RNAs fractionated by agarose gel electrophoresis (Fig. 3). A cognate new band should therefore be expected after RNase H-mediated cleavage. This was not the case (Fig. 4). We note, however, that such a species extending from the RNase H cleavage site to the U1A-BS should precisely migrate with the main RNA decay intermediate and thus be masked.
Importantly, deproteinization of the reporter was reduced in the ccr4 mutant strain, supporting the idea that it is responsible for the major fraction of the deproteinase activity and demonstrating that reporter mRNA deproteinization is indeed required to increase its decay. Indeed, Pop2 by itself, even tethered to its substrate, is unable to promote an efficient deproteinization. Consistently, there was no enhancement of the degradation of the reporter mRNA resulting from the targeting of Pop2 in the ccr4-deleted background (Fig. 5).

This analysis demonstrates that the new mRNA decay intermediate is not generated by the exosome. It is thus tempting to speculate that this new intermediate is generated by the tethered Pop2-Ccr4 deproteinase. The tethered complex would not stop when 10–15 A residues are left in the poly(A) tail but would proceed further to degrade the full poly(A) tail and part of the 3′-end of the mRNA body until it is blocked by the U1A-RBD. The absence of the new intermediate in the Ccr4-deleted strain is consistent with this possibility even though we cannot formally exclude that the accumulation of undegraded poly(A) on the reporter mRNA in this strain prevents the entry of another, as yet uncharacterized, 3′-5′-exonuclease.

**DISCUSSION**

In recent years numerous enzymes and regulatory factors involved in cytoplasmic mRNA decay have been identified (2, 3). However, the mechanisms allowing the tight regulation of this process remain largely unknown. Because deproteinization is often the rate-limiting step for mRNA decay (6), we have developed a targeting strategy to test whether this process may be regulated by association of the main yeast deproteinase with its substrate. Consistent with this idea we have shown that the targeted deproteinase specifically increases the rate of reporter mRNA decay. This is consistent with deproteinization being rate-limiting for mRNA degradation and thus that this step is a key target for regulatory factors. Furthermore, our results demonstrate that deproteinase recruitment may serve as a means to activate mRNA degradation in yeast. Although the exact mechanism by which deproteinase association with a target mRNA enhances its decay remains to be established, it is possible that this occurs through increased deproteinase processivity. However, other means to activate the Pop2-Ccr4 complex are also possible. These could include local stimulation of its nuclease activity, e.g. through transient post-translational modification (e.g. by phosphorylation or dephosphorylation), or modification of the substrate ribonucleoprotein allowing a more productive access of the enzyme.

Targeting of the Pop2-Ccr4 deproteinase also resulted in the production of a new, 3′-processed decay intermediate. This RNA fragment contained part of the 3′-UTR of the reporter transcript extending from the oligo(G) sequence to the U1A-binding site (Fig. 4B). Thus, this molecule derives from a decapped species that has been exonucleolytically degraded in the 5′-3′ direction by a nuclease that was blocked by the oligo(G) tract. We conclude that the deproteinization promoted by the targeted deproteinase also resulted in activation of degrading. However, in contrast to the main decay intermediate accumulating in wild-type cells, this new intermediate did not end with a 3′-oligo(A) tract following the polyadenylation site but rather contained a trimmed 3′-UTR (Fig. 4B). Surprisingly, this 3′-5′-exonuclease activity was not affected by a mutation of the exosomal subunit Rrp4/Dis3 but was completely inhibited in a mutant strain devoid of Ccr4. This suggests that this 3′-5′ trimming is not mediated by the exosome. Although the nuclease responsible for this trimming cannot be identified with certainty for the time being, our results indicate that the Pop2-Ccr4 deproteinase, which is required for this process, may be responsible for this degradation of the mRNA body. Consistent with this possibility, deproteinases have been shown to have a non-absolute substrate specificity, being able to remove residues other than A from RNA substrate, albeit at a reduced rate (14, 16). We note further that the 3′-UTR sequence removed from the substrate is, as for most yeast UTRs, likely to be unstructured, being relatively rich in A and U residues (respectively, 11 As and 19 Us over 36 nucleotides) and that this situation may also have contributed to its degradation by a deproteinase. Given that deproteinization is a prerequisite for the decay of the 3′-end of the mRNA body, further work will be required to identify definitively the enzyme involved in this process.

The plasmid system that we have built allows for a rapid targeting of factors involved in RNA metabolism. This tool could be used both for *in vitro* or *in vivo* analyses. Thus, fusion of U1A-RBD to Pop2 may now be used to alter the half-life of any target mRNA. Reciprocally, fusion of the U1A-RBD to Dcp2 or Pub1 did not result in altered degradation of the PGK1 reporter. Because Dcp2 is essential for normal cellular growth and because yeast cells expressing the Dcp2-U1A-RBD fusion as the only source of Dcp2 function grow well, we cannot attribute the lack of altered degradation of the reporter mRNA in these cells to a non-functional protein. Thus, either the Dcp2-U1A-RBD fusion is unable to bind the reporter mRNA or factors required to activate the Dcp2 protein are missing. Alternatively, the presence of a poly(A) tail and the bound Pab1 protein may inhibit decapping, as suggested by *in vivo* and *in vitro* studies (39–41). Indeed, in cells, Dcp2 appears unable to decap mRNA containing a poly(A) tail unless they contain a premature termination codon. Both possibilities are consistent with the observation that mRNA decay occurs in specialized cytoplasmic structures in mammalian and yeast cells. Further work should reveal the nature of the mechanism(s) mediating the tight control of decapping in eukaryotic cells.

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