The rate of mRNA decay is one factor that determines the expression in both prokaryotes and eukaryotes, and change in the scaffold protein GW182, Hedls (39) (also called Ge-1 [56]), homologs of the yeast P-body components plus eIF4E, eIF4E-T, or degraded in P bodies (8, 12). P bodies are also present in mammalian cells (2, 5, 18, 19, 29, 31), and the growing list of processing bodies, or P bodies) together with mRNA under- going degradation created a paradigm shift in our understand- ing of the process of mRNA decay (47). More recent work further simplified this picture, essentially dividing the cytoplasmic mRNP pool into two categories: mRNPs that are actively engaged with translating ribosomes and those that are stored or degraded in P bodies (8, 12). P bodies are also present in mammalian cells (2, 5, 18, 19, 29, 31), and the growing list of component proteins (reviewed in reference 17) includes orthologs of the yeast P-body components plus eIF4E, eIF4E-T, the scaffold protein GW182, Hedls (39) (also called Ge-1 [56]), RNA-associate protein 55 (55), and components of the RNA-induced silencing complex together with mRNA targeted for degradation by microRNAs (37, 46).

The best-characterized mammalian mRNA instability elements are the AU-rich elements, or AREs. These can be divided into several classes depending on the number and positioning of AUUUUA repeats and their impact on poly(A) shortening (reviewed in reference 4). AREs are targets for a number of RNA binding proteins, some of which exert a stabilizing effect (e.g., HuR [9]), some of which exert a destabilizing effect (e.g., tristetraprolin [TTP] and butyrate response factor 1 (BRF-1 [32, 48]) and at least one (Aup1 or hnRNP D) that can be stabilizing or destabilizing depending on the binding of different alternatively spliced isoforms (15, 54). In addition to progress made in identifying and characterizing proteins that bind to AREs (and other mRNA instability elements), we are beginning to understand the actual processes by which these mRNAs are degraded. Identification of 5’-3’ decay of unstable mRNAs in yeast was facilitated by the ability of a poly(G) tract to block progressive exonucleolytic degradation by Xrn1p (40). Similar approaches in mammalian cells have met with limited success; however, decapped and deadenylated mRNAs have been identified (13), suggesting that the same 5’-3’ decay process also occurs in mammalian cells.

A number of studies used in vitro systems to study ARE-mediated mRNA decay, and in these the ARE accelerates poly(A) shortening and decay of the mRNA body (20–23). Several of these reports also show that AREs and/or associated ARE-binding proteins recruit the exosome to degrade these unstable mRNAs with 3’-5’ polarity (10, 26, 42), and one report described a decapping activity similar to that seen in yeast Dep2 which is activated by an ARE (24). P bodies have also been identified as a major site for degradation of ARE-containing mRNA (17). As noted above, TTP and butyrate response factor 1 binding to the ARE activate decay, and Lykke-Andersen and Wagner (39) identified domains of these proteins responsible for this activation. In addition, they
showed that increased expression of Dcp2 enhances the decay of mRNA carrying the granulocyte-macrophage colony-stimulating factor (GM-CSF) ARE. Ferraiuolo et al. (19) identified eIF4E-T, the binding partner of eIF4E, in P bodies, and showed that eIF4E-T is essential for P-body integrity and that its knockdown by RNA interference (RNAi) stabilizes ARE-containing mRNAs. Using an RNAi screen for mRNA decay enzymes, Stockclin et al. (49) showed that β-globin mRNA carrying the GM-CSF ARE is stabilized following knockdown of two key components of P bodies, Xrn1 and Lsm1. Comparable data supporting 5'-3' decay for unstable mRNAs was obtained by introducing mRNAs with a number of novel cap analogs into cells (28).

In the course of our work on β-globin mRNA decay in erythroid cells, we sought a method to measure the decay rates of the 5', middle, and 3' portions of this mRNA that does not require primer binding to downstream sequences. This technique also had to be sufficiently sensitive to detect mRNA decay products without overexpressing the reporter mRNA. These criteria were met by the Invader RNA assay (Third Wave Technologies, Inc.) (16). The Invader RNA assay is a quantitative, isothermal assay in which a fluorescent signal accumulates linearly in proportion to the concentration of target RNA. Using this assay, we examined the polarity of decay of tetracycline-regulated β-globin mRNAs without the addition of any instability elements or with c-fos or GM-CSF AREs in the 3' untranslated region (3'-UTR). A similar decay pattern for each exon was seen for stable β-globin mRNA, with little evidence for polarity in its decay. Both AREs activated mRNA decay but with distinctly different polarities. The c-fos ARE activated simultaneous decay from both ends of the mRNA, whereas the GM-CSF ARE activated decay primarily from the 5' end. Unexpectedly, we observed that knockdown of Dcp2 reduces decay from the 3' end of mRNA carrying the c-fos ARE, and knockdown of PM/Scl-100 or Rrp41 reduces decay from the 5' end, indicating that the 5' and 3' decay pathways are functionally linked.

MATERIALS AND METHODS
Plasmid constructions and cell culture. Tetracycline-regulated plasmids expressing the β-globin gene without added instability elements (pTet-CMV

\[\text{max} \times g_{\text{max}} \text{ (maximum)} \times g_{\text{max}} \text{ (maximum) for 1 min at 4°C.} \]

that time point using antibodies to Dcp2, PM/Scl-100 and Rrp41. These data are presented in Fig. 2C, 3C, and 4E.

RNA isolation and Northern blotting. Adherent cells were washed twice with phosphate-buffered saline and harvested by scraping and centrifugation at 1,000 × g_{\text{max}} for 1 min at 4°C. The cell pellet from each 60-mm dish was resuspended in 200 μl of cytoplasmic extraction buffer (10 mM Tris-HCl, pH 8.0, 0.14 M NaCl, 1.5 mM MgCl\textsubscript{2}, 0.025% [v/vol] NP-40, 10 mM dithiothreitol, 30 units RNase OUT/100 μl) and incubated on ice for 10 min. Nuclei were removed from the lysed cells by centrifugation at 12,000 × g_{\text{max}} for 5.5 min at 4°C in a refrigerated microcentrifuge. RNA was recovered from the remaining cytoplasmic fraction using TRizol reagent (Invitrogen) or using the Absolutely RNA reverse transcription-PCR miniprep kit (Stratagene). Northern blotting was performed as described previously (7). DNA probes for both β-globin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized using the Random Primers DNA labeling system (Invitrogen). The template for synthesis of the β-globin probe was a 322-bp EcoRI fragment spanning a portion of exon 1, all of exon 2, and a portion of exon 3. The template for GAPDH probe synthesis was a 517-bp PCR product from CHOAA8 cDNA using primers CHOGAPDH3 (5'-AACCTTGGCATTGGAAAGGAC) and CHOGAPDH3 (5'-TTCTTACTCTTGGACGGACCATG).

Western blotting. Cells were harvested by scraping with phosphate-buffered saline and centrifugation at 1,000 × g_{\text{max}}, and the resulting pellets were resuspended in MPER mammalian protein extraction reagent (Pierce) plus protease inhibitor cocktail (Sigma) for isolation of total cell protein. Debris was removed by centrifugation, and protein concentration was measured using the biochinnic acid assay (Ferent). Fifteen to 25 μg protein was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. Membranes were blocked with rabbit antibodies to eIF4E-T, the binding partner of eIF4E, in P bodies, and GM-CSF ARE. Ferraiuolo et al. (19) identified lating factor (GM-CSF) ARE. Ferraiuolo et al. (19) identified


**TABLE 1. Oligonucleotides used in the Invader RNA assay**

<table>
<thead>
<tr>
<th>Probe set</th>
<th>Oligonucleotidea</th>
<th>Sequence (5’ to 3’)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>Probe</td>
<td>aacgagggctCTTCAATTCAC-NH2</td>
</tr>
<tr>
<td></td>
<td>Invader oligonucleotide</td>
<td>CAGGGCCCTACCCACAA</td>
</tr>
<tr>
<td></td>
<td>Stacking oligonucleotide</td>
<td>GUUGGCUGCAC</td>
</tr>
<tr>
<td></td>
<td>Arrestor oligonucleotide</td>
<td>GUGGAAUGAGAGGUCGC</td>
</tr>
<tr>
<td></td>
<td>FRET oligonucleotide</td>
<td>(6-FAM)-CAG-(EQ)-TGCTTCGTGG</td>
</tr>
<tr>
<td></td>
<td>SRT</td>
<td>CCAGGGAAAGCAGTGGTGCGCGTCCTGUA</td>
</tr>
<tr>
<td>Exon 2</td>
<td>Probe</td>
<td>aacgagggctCTAAAGGCACC-NH2</td>
</tr>
<tr>
<td></td>
<td>Invader oligonucleotide</td>
<td>GGTAGGCGCGGCACTCAA</td>
</tr>
<tr>
<td></td>
<td>Stacking oligonucleotide</td>
<td>GAGCCACUUUCUUGC</td>
</tr>
<tr>
<td></td>
<td>Arrestor oligonucleotide</td>
<td>GGUUCGUURUGUGGC</td>
</tr>
<tr>
<td></td>
<td>FRET oligonucleotide</td>
<td>(6-FAM)-CAG-(EQ)-TGCTTCGTGG</td>
</tr>
<tr>
<td></td>
<td>SRT</td>
<td>CCAGGGAAAGCAGTGGTGCGCGTCCTGUA</td>
</tr>
<tr>
<td>Exon 3</td>
<td>Probe</td>
<td>aacgagggctACCCAGAC-GNH2</td>
</tr>
<tr>
<td></td>
<td>Invader oligonucleotide</td>
<td>GTGATGCGCCACACACAGC</td>
</tr>
<tr>
<td></td>
<td>Stacking oligonucleotide</td>
<td>UUGGCCAGGAG</td>
</tr>
<tr>
<td></td>
<td>Arrestor oligonucleotide</td>
<td>CGUGCUGUGGUGG</td>
</tr>
<tr>
<td></td>
<td>FRET oligonucleotide</td>
<td>(6-FAM)-CAG-(EQ)-TGCTTCGTGG</td>
</tr>
<tr>
<td></td>
<td>SRT</td>
<td>CCAGGGAAAGCAGTGGTGCGCGTCCTGUA</td>
</tr>
</tbody>
</table>

a FRET, fluorescence resonance energy transfer; SRT, secondary reaction template.
b Lowercase bases indicate 5’ flap sequences. The cleavage base is indicated by boldface type. Underlined bases indicate 2’ O-methylated nucleotides. A 3’ NH2 was included to enhance assay performance. EQ, Eclipse quencher (Epoch Biosciences).

Invader RNA assay was performed. The net signal, mean, and standard deviation were calculated for each set of triplicate in vitro β-globin transcript standard samples. To determine which concentrations of β-globin transcript fell within the linear range of the assay, 10 tests were performed for each point on the standard curve and zero for each point on the standard curve and the next lowest value and the signal amplification change greater than zero was calculated. Only those data points with a t-test for both tests and changes (n-fold) of >0.5 greater than 1.15 were used to generate the standard curve which was plotted as relative light units (RLUs) versus attomoles of in vitro transcript on a semilogarithmic graph.

The Invader RNA assay is well suited to study the polarity of mRNA decay because the signal generated from each probe set is plotted as relative light units (RLUs) versus attomole of in vitro transcript on a linear metric before use. Precipitated. Each preparation was gel purified and quantified spectrophoto-

mRNA up to the ARE were prepared, and the three indicated on the figure met these criteria. Set 2 is located in the middle of exon 1, set 5 is located in the middle of exon 2, and set 8 is located toward the 5’ end of exon 3. For simplicity throughout the text, these will be referred to as the exon 1, 2, and 3 probes. Each Invader set has three components: a stacking oligonucleo- tide, a probe oligonucleotide, and an Invader oligonucleotide, the sum of which span a region of ~40 bases. The sequences of each probe set are presented in Table 1. The probe oligonucl eotide has a sequence that hybridsizes to the target RNA followed by a generic “flap” sequence. A stacker oligonucleotide binds immediately upstream of the probe to increase the melting temperature ($T_m$) of the probe, and the Invader oligonucleotide (shown beneath each sequence) hybridsizes down- stream of the probe with a 1-nm overlap at the junction of the “flap” sequence. This 1-nm overlap distorts the structure of the hybridized probe oligonucleotide, making it susceptible to cleavage by a modified form of $T_h$ polymerase (16) termed Cleavase. The reaction is run at the $T_m$ of the probe oligonucleotide so that flaps are generated in direct relationship to the concentration of the target mRNA. The flap serves as the Invader oligonucleotide in a second reaction that uses a common template and a probe bearing a quencher and fluorophores. Cleavage of the probe at the 1-nm overhang of this complex generates a fluorescent signal that is then read. Each experiment was done at least twice, and each Invader assay was performed on triplicate samples to obtain statistically significant results.

The decay of each reporter β-globin mRNA was studied using murine fibroblasts [LM(tk- tTA) cells] that express the tetracycline repressor protein. These cells were stably trans-
the amount of RNA that can be analyzed in each reaction mixture. Because of this, we could not do transcription pulse-chase and instead examined decay from steady state following the addition of tetracycline to cells in log-phase growth.

The first experiments examined the decay after transcription turnoff of \( \beta \)-globin mRNA lacking any added instability element (Fig. 2). When assayed by Northern blotting, this mRNA decays with a half-life of \( 8 \) h (Fig. 2A). However, a more complex pattern emerged when individual portions of this mRNA were quantified using the Invader assay (Fig. 2B). By definition, this mRNA is at steady state at the time tetracycline is added to the medium (time zero). At this point, the exon 2 and 3 probes detected 3 amol of mRNA in a 30-ng sample of total RNA, but the exon 1 probe reproducibly detected only 2 amol. The difference in mRNA detected with each of the probe sets was maintained throughout the time course, suggesting that the Invader assay identified a population within this slowly decaying mRNA that is decapped and in the process of 3'–5' decay.

Next we examined the impact of knocking down Dcp2 on the decay polarity of this stable mRNA. Western blots of each RNAi knockdown are presented in Fig. 2C. Transfecting cells with Dcp2 siRNA 48 and 24 h prior to transcription turnoff reduced this protein to 10% of control. When assayed by Northern blotting, the decreased level of Dcp2 had little impact on control \( \beta \)-globin mRNA during the first 4 h of decay; however, by 8 h it declined by 50% (Fig. 2D). A number of interesting differences became apparent when the decay of individual portions of this stable mRNA was studied by Invader assay (Fig. 2E). The first notable difference was that the steady-state levels of exons 1, 2, and 3 were half of those seen in Fig. 2B. Muhlrad and Parker (41) showed previously that loss of Dcp2 causes the steady-state level of reporter mRNA to decrease in a manner that is not linked to decay, and our results are consistent with their findings. Knockdown of Dcp2 also eliminated the quantitative difference seen at time zero between the amount of mRNA detected with probe to exon 1 versus to exons 2 and 3. In spite of this difference, knockdown of Dcp2 had no impact on the decay rates of exons 1, 2, and 3. Together, these data support the idea that stable mRNA decays incrementally faster from the 5' end than from the 3' end.

PM/Scl-100 is both nuclear and cytoplasmic (27), and the inhibition of 3'–5' decay of nonsense-containing mRNA by knockdown of this protein (34) was the first in vivo demonstration of a role for the exosome in the decay process. An siRNA that was shown previously to effectively target PM/Scl-100 (52) reduced this protein by 90% in cells expressing each of the target mRNAs (Fig. 2C). When the decay of stable \( \beta \)-globin mRNA in cells treated with this siRNA was assayed by Northern blotting, there was a modest decrease in half-life from 8 h to 5.5 h (Fig. 2F). As one might anticipate, results with the Invader assay showed there was little impact of PM/Scl-100 knockdown on 3'–5' decay (Fig. 2G). As in Fig. 2B, there was less of exon 1 (0.9 amol) at time zero than exons 2 (1.5

**FIG. 1.** Locations of Invader probe sets on \( \beta \)-globin mRNA. The sequence of human \( \beta \)-globin mRNA is shown with exons 1 and 3 in gray, exon 2 in white, and the c-fos ARE in black. In the constructs used here, the \( \beta \)-globin polyadenylation signal has been replaced by a dominant synthetic poly(A) site (SPA [35]). Each of the Invader probe sets detailed in Table 1 is shown in its corresponding position on \( \beta \)-globin mRNA. In each set, the stacker oligonucleotide is on the left, the probe oligonucleotide with the unpaired flap is shown in thick black lines, and the Invader oligonucleotide is shown underneath the sequence with the overlapping base drawn at an angle.
amol) and 3 (1.4 amol), and this quantitative difference was maintained throughout the time course. We noted above that decay does not appear to be as important as $5'_{/H11032}$ decay for this mRNA, and the minor effect of PM/Scl-100 knockdown observed here supports this observation. Overall, these results indicate that a stable mRNA decays from both ends with $5'_{/H11032}$ decay proceeding incrementally faster than $3'_{/H11032}$ decay.

The c-fos ARE simultaneously activates $5'$ and $3'$ decay. The AREs are classified by the number and nature of AUUUA repeats and the sequence context where they reside (3, 4). The class I AREs, which include c-fos, are characterized by multiple separate AUUUA elements in a U-rich context. These mRNAs undergo synchronous poly(A) shortening prior to decay of the mRNA body (53). Northern blot analysis showed that the c-fos ARE reduced the half-life of $\beta$-globin mRNA from 8 h to 3 h (Fig. 3A). However, a more complex picture emerged when the decay of each exon was quantified by Invader assay (Fig. 3B). After transcription turnoff, exons 1 and 3 decayed simultaneously, each with a half-life that matches the 3-h half-life determined by Northern blotting. This was followed by decay of exon 2, indicating that the c-fos ARE simultaneously activates decay from both the $5'$ and $3'$ ends of the mRNA.

The roles of the decapping and $5'_{/H11032}$-$3'_{/H11032}$ decay pathways were examined as in Fig. 2C and D by knockdown of Dcp2. When decay was assayed by Northern blotting, the loss of Dcp2 had no impact on c-fos mRNA decay (Fig. 3D). Again, a more complex pattern emerged when each exon was quantified by Invader assay (Fig. 3E). As seen in Fig. 2E, knockdown of Dcp2 caused a twofold decrease in the steady-state level of reporter mRNA. At time zero, there was twice as much exon 1 and 2 than exon 3, indicating that Dcp2 knockdown inhibited $5'$ decay of c-fos mRNA but had little impact on ARE-stimulated $3'$ decay. Exon 3 decayed linearly until 2 h, at which time it reached the limit of detection for this experiment and plateaued. This was matched by decay of exons 2 and 1, with both...
disappearing with almost linear kinetics for 4 to 6 h after transcription turnoff. There are two possible interpretations for the similar decay of exons 1 and 2. In one, 5'-H11032 decay is inactivated and the delayed but simultaneous loss of exons 1 and 2 is due to processive 3'-H11032 decay by the exosome. In the other, 5'-H11032 decay is slowed but not inactivated and the simultaneous decay of exons 1 and 2 results from a combination of this and exosome-mediated 3'-H11032 decay. While we favor the former, at this time these cannot be formally distinguished.

PM/Scl-100 was again targeted by RNAi to characterize the 3'-H11032 decay of c-fos mRNA. Knockdown of PM/Scl-100 increased the reporter mRNA half-life measured by Northern blotting from 3 h to 4 h (Fig. 3F). While this might be interpreted to be a minor effect, it masked major changes in the overall decay process. The most striking change was in the decay of exon 3, whose half-life changed from 3 h in untreated cells to essentially no decay (Fig. 3G). This is identical to that seen for control mRNA in Fig. 2G following knockdown of PM/Scl-100, indicating that loss of this exosome component effectively inactivated 3' decay. In the absence of 3' decay, the curves generated with the exon 1 and 2 probes show the contribution of 5' decay to the turnover of c-fos mRNA. While exon 1 still decayed more rapidly than exon 2 and both decayed faster than the corresponding exons of control mRNA in Fig. 2, the overall rate of 5' decay was slower than that seen in untreated cells (Fig. 3B). Notably, exon 1 decayed with the same half-life as that determined by Northern blotting, confirming this as the rate-limiting step in the absence of 3' decay. Although P bodies are thought to be the sites where 5'-H11032 decay occurs, neither the transfection process itself nor knockdown of PM/Scl-100 reduced the size or number of these foci (data not shown).

The GM-CSF ARE preferentially activates 5'-3' decay. The class II AREs have multiple overlapping AUUUA repeats (3, 4), and unlike class I AREs, mRNAs with these elements undergo asynchronous poly(A) shortening (53). To determine whether the bidirectional decay observed with the c-fos ARE is a general property of ARE-stimulated decay, we examined the decay polarity of β-globin mRNA carrying the class II GM-CSF ARE. The Northern blot in Fig. 4A shows that GM-CSF

![Graphs and images](http://mcb.asm.org/00000003/images/3f.jpg)
mRNA decays with a half-life of 1.5 h. In contrast to the results seen with the c-fos ARE, mRNA with the GM-CSF ARE decays primarily with 5'-3' polarity (Fig. 4B). Exons 1 and 2 decay rapidly and almost simultaneously with the 1.5-h half-life seen by Northern blotting, followed by exon 3.

The stabilization of c-fos mRNA exon 3 after knockdown of PM/Scl-100 confirmed the bidirectional nature of its decay process but also indicated that 5' decay was not sufficiently processive to completely degrade the body of this mRNA. To determine whether there is a 3' decay component to the loss of GM-CSF mRNA, we examined the impact of knocking down Rrp41, the catalytic subunit of the exosome (38). Results in Fig. 4E show that transfecting these cells twice at 24-h intervals with Rrp41 siRNA reduced its level to 10% of control. A control (GL2) siRNA had no impact on the amount of Rrp41 or on the preferential 5'-3' decay of this mRNA (Fig. 4F).

Knockdown of Rrp41 significantly reduced the decay of exon 3, thus confirming a lesser but nonetheless significant role for the exosome in the decay of this mRNA.

DISCUSSION

In yeast, unstable mRNAs decay with 5'-3' polarity and the identification of focal concentrations of enzymes involved in decapping and 5'-3' decay (P bodies) in yeast and mammalian cells led to the general conclusion that these are the primary sites of mRNA decay. Most of the support for this concept in mammalian cells came from coprecipitation experiments that identified complexes containing decay-promoting proteins with proteins found in P bodies and from RNAi knockdown experiments where loss of P-body components stabilized ARE-containing mRNAs (19, 39, 49). The recent focus on P bodies contrasts with results from in vitro experiments that identified complexes containing decay-promoting proteins with proteins found in P bodies and from RNAi knockdown experiments where loss of P-body components stabilized ARE-containing mRNAs (19, 39, 49). The recent focus on P bodies contrasts with results from in vitro experiments that identified complexes containing decay-promoting proteins with proteins found in P bodies and from RNAi knockdown experiments where loss of P-body components stabilized ARE-containing mRNAs (10, 42, 51). Here we describe a new approach using the Invader RNA assay that allowed us to quantify the decay of individual portions of a β-globin reporter mRNA independent of structural elements within the mRNA [e.g., poly(G) tracts] or RNAi knockdown of...
proteins involved in the decay process. By comparing these results with those obtained by Northern blotting, we were able to define the relative contribution of 5’ and 3’ pathways to the overall process of mRNA decay. All of the work described here used stable cell lines that express low levels of each reporter mRNA in order to avoid overexpression artifacts sometimes seen with transient transfection. This precluded the use of transcription pulse-chase experiments, since the amount of mRNA induced in the short intervals tested was at the limit of detection for the Invader assay. Instead we focused on decay from steady state after turning off transcription by adding tetracycline to the medium, and the first results showed that there is little evidence for differences in the decay rates of any of the exons of stable β-globin mRNA (Fig. 2). A different picture emerged with the Invader assay used to study the decay of two different ARE-containing mRNAs. In both cases, adding an ARE activated mRNA decay; however, c-fos mRNA decays simultaneously from both ends (Fig. 3B), whereas GM-CSF mRNA primarily undergoes 5’-3’ decay (Fig. 4B). Interestingly, evidence for simultaneous 5’ and 3’ decay of the same mRNA was seen previously in trypanosomes (30).

The individual contributions of the 5’ and 3’ decay pathways to the patterns observed with the Invader assay was supported by results using RNAi to reduce the level of Dcp2, PM/Scl-100, or Rrp41. Deleting Dcp2 from yeast causes a generalized decrease in reporter mRNA that is independent of mRNA decay (41), and we observed a similar phenomenon following knockdown of Dcp2. In spite of this, knockdown of Dcp2 also reduced 5’ decay without altering 3’ decay (Fig. 3E). PM/Scl-100 is the mammalian ortholog of Rrp6, a protein generally considered to be part of the nuclear exosome. When we began this work, it was the only exosomal protein whose knockdown had been shown to functionally impact mRNA decay (34), so this was targeted here. Others identified PM/Scl-100 in the cytoplasm (27, 34) and our data showing its knockdown stabilizes c-fos exon 3 points to a role for this protein in mRNA decay. Rrp41 was recently identified as the catalytic subunit of the mammalian exosome (38), and its knockdown caused a similar stabilization of exon 3 of GM-CSF mRNA (Fig. 4D).

A high-throughput approach is needed to more thoroughly characterize the contributions of the different classes of these elements to activating each of the major decay pathways. Nevertheless, we can draw a number of conclusions from our results. First, each of the major decay pathways participates to some extent in the turnover of unstable mRNAs. As noted above, in vitro decay experiments supported a predominant role for the exosome (10, 26, 42), whereas RNAi knockdown and coprecipitation experiments supported a predominant role for decapping and 5’-3’ decay (39, 49). The latter studies used a β-globin reporter mRNA carrying the GM-CSF ARE similar to the GM-CSF reporter used here, and our results demonstrating 5’-3’ polarity in the decay of this mRNA nicely complement their findings. Second, the two elements examined here represent the class I (c-fos) and class II (GM-CSF) AREs and while these have different effects on poly(A) shortening (53), our results show they also define the manner by which mRNA is degraded. An important area for future study will be to determine how the various ARE-binding proteins guide the relative contribution of each of the major decay pathways. The third conclusion is that the 5’-3’ and 3’-5’ decay pathways are functionally linked. While there was no discernible change in 3’ decay after knockdown of Dcp2, the 5’ decay of both c-fos and GM-CSF mRNAs was slowed by knockdown of PM/Scl-100 or Rrp41. This linkage is supported by results of a comprehensive yeast two-hybrid screen of human proteins involved in mRNA decay showing Skl2 interacts with both Xrn1 and PM/Scl-100 (33). Additional support for this is seen in the coprecipitation of Rrp4 with Dcpl, Dcp2, and Xrn1 by TTP and BRF-1 (39) and the inhibition of GM-CSF ARE-stimulated mRNA decay by knockdown of PM/Scl-75 (49). TTP and BRF-1 possess specific activating domains, and it is tempting to speculate that these domains coordinate the 5’-3’ and 3’-5’ decay processes observed here.

Finally, the data presented here indicate it is an oversimplification to think that mRNA decay is restricted to P bodies. Several lines of evidence point to P bodies as sites where mRNAs accumulate during microRNA-mediated silencing (1, 36, 37, 44). In addition, there is evidence from yeast (8, 12) and mammalian cells (6) that mRNAs can reversibly transit into and out of P bodies. Each of the major exonuclease decay pathways participates in the decay of ARE-containing mRNA, and since the exosome is not present in P bodies (1), it is difficult to envision how, for example, c-fos mRNA can undergo simultaneous 5’ and 3’ decay if P bodies are the sole engine of mRNA decay. While knockdown of PM/Scl-100 inactivated 3’ decay, it had had no visible impact on the number and size of P bodies (data not shown). On the basis of our results and those of others linking the exosome to decapping (45, 51), we propose that the exosome and submicroscopic complexes of enzymes involved in decapping and 5’ decay are linked by a dynamic interaction that facilitates and coordinates the decay of unstable mRNAs.

ACKNOWLEDGMENTS

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REFERENCES


