MOLECULAR AND CELLULAR BIOLOGY, Jan. 1997, p. 398–406 Vol. 17, No. 1
0270-7306/97/$04.00+0

The Poly(A) Tail Inhibits the Assembly of a 3′-to-5′ Exonuclease in an In Vitro RNA Stability System

LANCE P. FORD, PARAMJEET S. BAGGA, AND JEFFREY WILUSZ*

Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, Newark, New Jersey 07103

Received 3 September 1996/Accepted 21 October 1996

We have developed an in vitro system which faithfully reproduces several aspects of general mRNA stability. Poly(A)$^-$ RNAs were rapidly and efficiently degraded in this system with no detectable intermediates by a highly processive 3′-to-5′ exonuclease activity. The addition of a poly(A) tail of at least 30 bases, or a 3′ histone stem-loop element, specifically stabilized these transcripts. Stabilization by poly(A) required the interaction of proteins with the poly(A) tail but did not apparently require a 3′ OH or interaction with the 5′ cap structure. Finally, movement of the poly(A) tract internal to the 3′ end caused a loss of its ability to stabilize transcripts incubated in the system but did not affect its ability to interact with poly(A) binding proteins. The requirement for the poly(A) tail to be proximal to the 3′ end indicates that it mediates RNA stability by blocking the assembly, but not the action, of an exonuclease involved in RNA degradation in vitro.

The relative stability of RNA transcripts plays a key role in determining their steady-state levels as well as the rate of mRNA induction following a transcriptional stimulus (30). Mutations which affect transcript stability can have a very significant impact on regulated gene expression (26, 33). The mechanism of regulated turnover of mammalian mRNA, however, is largely unknown. Terminal structures, namely, the 5′ cap and 3′ poly(A) tail, serve as general stabilizing elements found on most mRNAs (7, 14). Several internal sequences, such as an AU-rich element (10) or nonsense codons (5), which functionally shorten the half-lives ($t_{1/2}$) of mRNAs have been identified. Furthermore, an internal element which stabilizes α globin mRNA has recently been identified (38). Elucidating how the general and regulatory elements interact to determine the functional $t_{1/2}$ of mRNAs is vital to understanding the mechanism of RNA stability as a regulator of gene expression.

The poly(A) tail, a posttranscriptional modification of the 3′ end of all nonhistone mRNAs, is initially formed as a 150- to 200-base homopolymer (19) which assembles multiple molecules of a poly(A) binding protein (PABP) (13). The tail is a dynamic structure which serves as a mediator through which several important cellular processes including the initiation of translation (31), nucleocytoplasmic transport (18), and mRNA stability (7), are regulated. Most mRNAs are rapidly degraded following deadenylation of their 3′ ends to approximately 30 adenylate residues (8, 21, 27, 34). Many destabilizing elements appear to act by increasing the rate of deadenylation (11). The disruption of poly(A) tail function, therefore, appears to be the rate-limiting step in mRNA turnover. Following deadenylation, mRNA degradation can occur through a variety of exo- and/or endonucleolytic pathways (4). The difficulty in identifying intermediates in mammalian mRNA turnover has significantly hindered attempts to probe further into mechanistic aspects of the turnover process.

Mechanistic questions in mammalian systems are usually best approached by using in vitro systems which can be fractionated and reconstituted. In vitro systems have been highly successful in providing clues to general mechanistic aspects of gene expression (16). Regulated aspects of gene expression at the posttranscriptional level, on the other hand, have been difficult to reproduce in cell-free systems, and limited success has been attained in only a few instances (23, 24). In spite of this, much of the effort to develop valid in vitro systems designed to study mRNA stability has focused on mimicking regulated aspects of differential mRNA turnover (reviewed in reference 29). While several systems have successfully reproduced aspects of in vivo mRNA turnover, they have, on the whole, been difficult to replicate in other laboratories.

In this study we describe an in vitro system using standard cellular extracts which accurately and efficiently reproduces a key aspect of general RNA turnover, namely, the effect of poly(A) tail length on mRNA stability. We have used this system to determine that the major pathway of RNA turnover in vitro involves a highly processive 3′-to-5′ exonuclease. The poly(A) tail in conjunction with poly(A) binding proteins functions to inhibit the assembly, but not the activity, of the enzyme complex responsible for RNA turnover. These data provide important insights for models of general and regulated turnover, as well as an experimentally tractable system for future studies.

MATERIALS AND METHODS

Plasmids. pLFMyc-1, which contained a 556-bp fragment of the 3′ untranslated region (3′ UTR) of the human c-*myc* gene (position 7217 to 7773), was constructed as follows. Oligonucleotide primers representing positions 7217 to 7241 and 7773 to 7748, which possessed a 5′ *Bam*HI or *Hin*dIII site, respectively, were used to amplify the indicated region from a cDNA clone of c-Myc (pSVPc-MYC [20], obtained from M. B. Small) by PCR. A 556-bp fragment, obtained after digestion of the amplified product with *Bam*HI and *Hin*dIII, was cloned into the corresponding sites of pGEM4 (Promega). An *Ssp*I site (AATAAT), located at position 7483, was changed to an *Hpa*I site (TAACAT) by megaprimer mutagenesis with the appropriate primers (1). The resulting plasmid served as the source of template for production of the 587-base myc RNA following *Hin*dIII digestion. Transcription of plasmids linearized with *Pvu*II gave a 634-base RNA (mycNS).

The construction of pSVL-GEM, which contains the 3′ UTR of the simian virus 40 (SV40) late transcription unit (nucleotides 2533 to 2682), has been described previously (39). *Hin*dIII digestion generated a template which produced the SV or SVA0 RNA. The construction of pSVE-G, which contains the 3′ UTR of the SV40 early region followed by a polylinker sequence containing a 14-base G-rich sequence, was as described previously for a UUUUU insertion (41). *Hin*dIII-linearized plasmid yielded a 171-base RNA following in vitro

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, 185 South Orange Ave., Newark, NJ 07103. Phone: (201) 982-5218. Fax: (201) 982-3644. E-mail: wilusz@umdnj.edu.

TABLE 1. Sense strands of oligonucleotide pairs used in constructions in this study

Construct	Starting plasmid	Oligonucleotide[a]
SVA10	pSVL-GEM	5′-AGCTAAAAAAAAAAATATTGAGGTGCTCGAGGT
SVA20	pSVL-GEM	5′-AGCT(A_{10})AAAAAAAAAATATTGAGGTGCTCGAGGT
SVA30	pSVL-GEM	5′-AGCT(A_{20})AAAAAAAAAATATTGAGGTGCTCGAGGT
SVA60	pSVL-GEM	5′-AGCT(A_{50})AAAAAAAAAATATTGAGGTGCTCGAGGT
mycA60	pLFMYC	5′-AGCT(A_{50})AAAAAAAAAATATTGAGGTGCTCGAGGT
mycHis	pLFMYC	5′-AGCTCTACCAATAAGAGGCCCTTTTCAGGGCCCCTGGAGTGCTCGAGGTA
SVE-G poly(A)$^+$	pSVE-G	5′-AGCT(A_{20})AAAAAAAAAATATTGAGGTGCTCGAGGT
SVintA60	pSVL-GEM	5′-AGCT(A_{50})AAAAAAAAAATATTGAGGTGCTCGAGGT
SVA30>P	pSVL-GEM	5′-AGCT(A_{30})GUCUGCUCGAGGTA
SVA30int	pSVL-GEM	5′-AGCT(A_{20})AAAAAAAAAATATTGGTACCATGCATATGGG

[a] The underlined bases indicate the restriction enzyme site(s) (if any) used to create templates for in vitro transcription. The underlined *Ssp*I, *Kpn*I, *Nsi*I, and *Nde*I sites of the SVA30int oligonucleotide were used to create RNAs with poly(A) tails located 1, 5, 11, and 21 bases internal to the 3′ end. A_{10}, A_{20}, A_{30}, and A_{50} refer to poly(A) tracts 10, 20, 30, and 50 bases long, respectively.

transcription [SVE-G Poly(A)$^-$]. pGRS, which contains a 14-base G-rich sequence inserted into the pGEM3 polylinker region, was constructed as previously described (28). In vitro transcription of *Hin*dIII-digested DNA gave a 51-base transcript (GRS).

Alterations of 3′ ends by a ligation-PCR approach. Variant RNAs containing additional sequences on their 3′ ends were generated from the starting plasmids described above by a ligation-PCR approach. Briefly, pairs of oligonucleotides were generated which had the following general structure: *Hin*dIII linker-desired sequence-restriction site-segment for PCR priming. Oligonucleotide pairs were ligated to the indicated plasmid DNA which had been linearized with *Hin*dIII. Ligated products were amplified in PCRs using an SP6 primer (5′-CATACGA TTTAGGTGACACTATAG) and the appropriate downstream primer (5′-TAC CTCGAGCACTC, 5′-TACCTCGAGCAGAC, or 5′-CCCATATGCATGGT A). Amplified products were purified on Centricon 100 columns, cut with the appropriate restriction enzyme, and used as templates for in vitro transcription reactions. Table 1 lists the sense strands of oligonucleotide pairs used in these constructions.

RNA production. All RNAs were produced in vitro with SP6 polymerase as described previously (40). Transcripts were purified on 5% acrylamide–7 M urea gels prior to use. SVA0 and SVA30 RNAs whose 3′ ends were modified to 2′,3′ cyclic phosphates (SVA0>P and SVA30>P RNAs, respectively) were generated by site-specific cleavage reactions as previously described (17) with hammerhead ribozymes prepared by SP6 transcriptions using the following oligonucleotides and their appropriate complementary strands: SV>P, 5′-ATTTAGGTGACAC TATAGAATACACCCTGCAGGTCCTGATGAGTCCGTGAGGACGAAAC TCTAGAGG, and SVA30>P, 5′-ATTTAGGTGACACTATAGAATACACTA CCTCGAGCACTGATGAGTCCGTGAGGACGAAACTTTTTTTT.

In vitro stability assays. Nuclear salt wash extracts were prepared from HeLa spinner cells grown in Joklik's modified minimal essential medium (JMEM) containing 10% horse serum as described by Dignam et al. (12). ^{32}P-labeled, gel-purified RNAs were added to the in vitro stability reaction mixtures containing 3% polyvinyl alcohol, 1 mM ATP, 20 mM phosphocreatine, 12 mM HEPES (pH 7.8), 12% glycerol, 60 mM KCl, 0.3 mM dithiothreitol, and 60% dialyzed extract. Reaction mixtures were incubated at 30°C for the indicated times, and this was followed by the addition of 400 μl of a buffer containing 400 mM NaCl, 25 mM Tris (pH 7.5), and 0.1% sodium dodecyl sulfate (SDS) and immediate phenol extraction. RNAs were concentrated with ethanol and analyzed on acrylamide gels containing 7 M urea. Bands were visualized by autoradiography or phosphorimaging. All quantitation was performed with a Molecular Dynamics PhosphorImager.

Competition assays were performed by adding the indicated amounts of cold ^{m7}GpppG cap analog or homopolymer RNAs (Pharmacia) directly to the in vitro stability reaction mixtures prior to incubation.

UV cross-linking assays. Equal molar amounts of RNAs labeled to the same specific activity at adenylate residues were incubated in the in vitro stability system and protein-RNA interactions were analyzed by UV cross-linking as described previously (40). Briefly, reaction mixtures were adjusted to 0.88 mM EDTA (to stabilize all RNA substrates to allow for accurate comparisons), irradiated with a germicidal light, and treated with a mixture of RNases A, T_1, and T_2 to generate cross-linked RNA binding proteins containing small radioactive RNA oligomers. Cross-linked proteins were analyzed on 10% acrylamide–SDS gels and visualized by autoradiography.

RESULTS

An in vitro RNA stability system that reproduces general aspects of RNA turnover. The key problem in the development of an in vitro system to study RNA stability lies in distinguishing degradation due to contaminating RNases from the true processes involved in mRNA turnover. Many previous efforts have addressed this issue by attempting to reproduce regulated mRNA turnover in vitro (29). In general, reproducing regulated events of RNA processing or transcription in vitro has proven difficult and has met with only limited success. Our approach to develop an in vitro RNA stability system was to try to reproduce general observations regarding mRNA turnover using cell extracts which have proven successful in reconstituting other RNA processing events. We were unable to achieve satisfactory results with cytoplasmic extract preparations due to low levels of activity and/or high levels of nonselective background RNases. We obtained the best results with nuclear salt wash extracts prepared according to the method of Dignam et al. (12). The hypotonic lysis and low-speed-pelleting step involved in the isolation of the nuclear fraction provide significant cytoplasmic contamination (data not shown; also note the presence of the cytoplasmic PABP in Fig. 4). This observation suggested that in addition to nuclear factors, several of the components of the cytoplasmic mRNA degradation machinery could be present in the nuclear salt wash fraction and that this extract may be exploitable to study mechanistic aspects of mRNA stability.

The first general criterion we used to develop an in vitro RNA stability system was to reproduce one well-defined aspect of mRNA degradation observed in vivo. Following deadenylation, many mammalian mRNAs are rapidly and efficiently degraded in the cell with no detectable intermediates (34). We incubated RNAs derived from the 3′ UTR of the c-*myc* and SV40 late mRNAs with salt wash extracts in the in vitro stability system as described in Materials and Methods. As seen in Fig. 1, both RNAs were rapidly and efficiently degraded in this system, with no detectable intermediates ($t_{1/2}$ = approximately 4 min). RNA turnover required divalent cations (EDTA sensitive), proteins (proteinase K sensitive), and added ATP (data not shown). Furthermore, turnover was not affected by the addition of human placental RNase inhibitor, suggesting that common RNase A family enzymes were not active in the system (data not shown). These data suggest that the turnover of RNA in our in vitro system may mimic the general mechanism of in vivo mRNA turnover.

The addition of poly(A) to the 3′ end stabilized RNAs in the in vitro system. Since deadenylation is a prerequisite for the degradation of many mRNAs, we tested if the addition of a poly(A) tail of sufficient length would stabilize RNAs in our in vitro system. Using the ligation-PCR approach described in Materials and Methods, we added poly(A) tails of 10, 20, 30, or 60 bases to the 3′ end of the normally unstable SV RNA and assessed the fate of the variant transcripts in the in vitro sta-

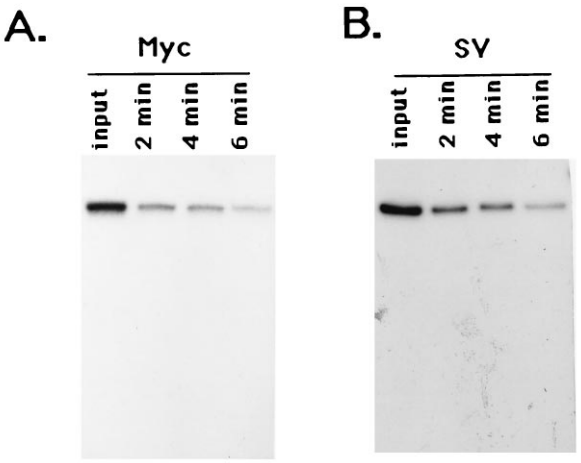

FIG. 1. RNAs are rapidly and efficiently degraded in an in vitro RNA stability system. Transcripts containing portions of the 3′ UTR of the c-*myc* (A) or SV40 late (B) mRNAs were incubated for the indicated times in an in vitro RNA stability system. Reaction products were purified and analyzed on 5% acrylamide gels containing 7 M urea.

bility system. As seen in Fig. 2, derivatives of SV RNA containing poly(A) tails of 0 or 10 bases (SVA0 and SVA10, respectively) were quickly degraded, with no detectable intermediates. The addition of 20 A's to the 3′ end of the SV RNA, however, resulted in a transcript of intermediate stability, while a poly(A) tail of 30 or 60 bases effectively stabilized the SV transcript for at least 3 h (maximum incubation times tested; data not shown). Poly(A)-mediated RNA stabilization could be generalized to other RNAs, as similar data were obtained for increasing lengths of poly(A) tail on the 3′ end of the myc RNA (Fig. 3, compare lanes mycA0 and mycA60; also data not shown). From these data we conclude that the addition of 30 or more adenylates to the 3′ end stabilizes transcripts in our in vitro stability system, while RNAs with shorter poly(A) tails are subject to rapid degradation. This faithfully mimics the relationship between the presence of an adequately long poly(A) tail and RNA decay observed in vivo (7).

We performed several experiments to test if the stabilizing effect of poly(A) on RNAs incubated in our in vitro system was specific. First, we determined whether adding 47 nonspecific bases to the 3′ end of the myc RNA would have a stabilizing effect similar to that by adding poly(A). As seen in Fig. 3, the addition of nonspecific sequence to the 3′ end failed to stabilize the transcript (lanes mycNS). These data argue that the effect of poly(A) we observed on RNA stability was sequence dependent and not simply the result of elongating the 3′ end of the transcript. In a second experiment, we tested whether the other known 3′ end mRNA stabilizing element, the stem-loop structure of histone mRNAs (22), would influence the turnover of RNAs in our in vitro system. Using ligation-PCR, we placed a histone stem-loop element at the 3′ end of the myc RNA. As seen in Fig. 3, this structure stabilized the myc RNA as efficiently as the addition of a 60-base poly(A) tail at all times tested (compare lanes mycA60 and mycHis). Similar data were obtained by adding the histone stem-loop onto the 3′ end of the SV transcript (data not shown). We conclude that the stabilizing effect of adding sequences to the 3′ end of transcripts in our system is specific for natural stabilizing elements, such as poly(A) or the histone mRNA stem-loop.

Previous in vitro observations by Ross and colleagues have shown that PABP plays an important role in the stabilizing function of the poly(A) tail (6). In order to access the role of poly(A) binding proteins in our system, we performed competition experiments using poly(A) and other homopolymer RNAs. As seen in Fig. 4, the addition of poly(A), but not an equivalent amount of poly(I) or poly(C), caused the rapid degradation of the normally stable SVA60 RNA. UV cross-linking studies using SVA60 RNA labeled at A residues showed that two proteins specifically cross-linked to the poly(A) tail in our in vitro system (Fig. 4B, bands denoted by arrows). Based on their apparent molecular weight and requirement for a poly(A) tail for cross-linking to RNAs, we have identified the upper band (approximately 70 kDa) as the abundant cytoplasmic PABP (15) and the lower band (approximately 48 kDa) as the nuclear PABII protein (37). The addition of 500 ng of poly(A) competitor RNA, but not poly(C), resulted in the specific competition of these two bands. The low-level competition for poly(A) binding protein-RNA interactions seen with poly(I) was nonlinear and could not be competed further even with 5,000 ng of poly(I) (data not shown). The amount of cold poly(A) required to fully compete with the poly(A) tail of SVA60 RNA for poly(A) binding factors was equivalent to the amount of cold poly(A) required to destabilize the transcript (data not shown). The ability of poly(A) binding proteins to efficiently cross-link with RNAs containing

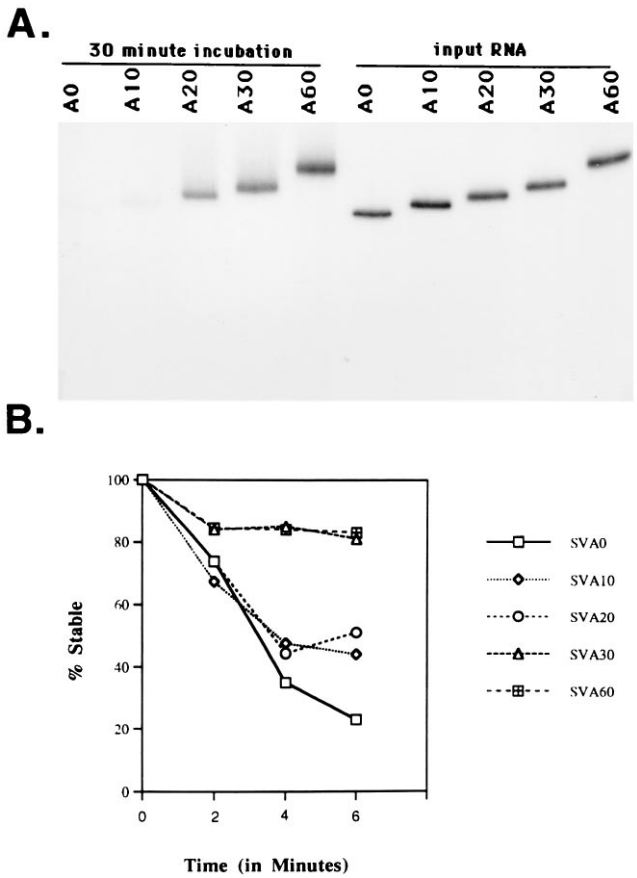

FIG. 2. The addition of 30 or more adenylates to the 3′ end effectively stabilized transcripts in the in vitro RNA stability system. (A) Variants of the SV RNA which contained 0, 10, 20, 30, or 60 adenylates at their 3′ ends (lanes A0, A10, A20, A30, and A60, respectively) were analyzed on a 5% acrylamide–urea gel before (input RNA) or after a 30-min incubation in the in vitro stability system. (B) Quantitative analysis of three experiments using the same transcripts described in panel A. The graph shows the averages of the values obtained. The standard errors of the means were determined for each RNA as follows: SVA0, 2.1%; SVA10, 5.6%; SVA20, 10.7%; SVA30, 13.5%; and SVA60, 6.4%.

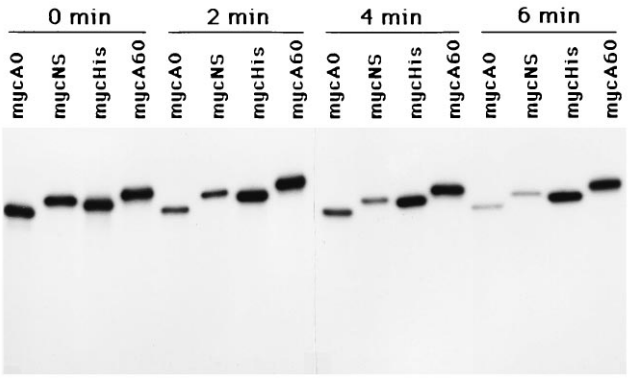

FIG. 3. RNAs can be stabilized in vitro by natural 3′ end stabilization elements but not by random sequences. Variants of a transcript containing the 3′ UTR of c-*myc* (mycA0) were prepared which contained one of the following 3′-end sequences: 47 bases of nonspecific, plasmid-derived sequence (mycNS); a stem-loop structure found at the 3′ end of histone mRNAs (mycHis); and a 60-base poly(A) tract (mycA60). Transcripts were incubated for the indicated times in the in vitro stability system, and reaction products were purified and analyzed on 5% acrylamide gels containing 7 M urea.

poly(A) tails at their 3′ ends of 0, 10, 20, 30, and 60 bases also directly correlated with relative transcript stability (data not shown). We conclude that *trans*-acting factors, most likely the well-characterized PABP or PABII, are required for poly(A) tail-mediated RNA stability in our in vitro system.

We, therefore, have developed an in vitro RNA stability system which faithfully reproduces several of the known properties of general mRNA turnover. First, RNAs are turned over in our system quickly and efficiently with no accumulation of intermediates. Second, a poly(A) tail of 30 or more nucleotides functionally stabilizes RNA in our system. Shorter poly(A) tails are less efficient or totally lack the ability to stabilize transcripts. Finally, poly(A) tail-mediated RNA stabilization required *trans*-acting factors, most likely one or more poly(A) binding proteins. Our preliminary studies have not detected a deadenylation activity in the in vitro system or regulated turnover which requires known destabilizing sequences. Since we are using fractionated cell extracts, however, it may be possible to reconstitute these activities in future studies. In its present form, this in vitro system provides a valid assay to address questions regarding mechanisms of poly(A)-mediated RNA stabilization.

A 3′-to-5′ exonuclease is the major mechanism of RNA turnover in vitro. The first mechanistic question we addressed using the in vitro RNA stability system was the identification of the major pathway of RNA turnover by attempting to characterize intermediates in the process. Previous studies have successfully used the insertion of a poly(G) tract in constructs to isolate mRNA degradation intermediates in vivo in *Saccharomyces cerevisiae* (25, 36). We tested whether the insertion of a 14-base G-rich tract into a normally unstable RNA substrate (GRS) would allow the identification of transient intermediates during RNA turnover in our in vitro system. The variant transcript was 5′ end labeled through in vitro capping using [^{32}P]GTP and incubated in the in vitro stability system. As seen in Fig. 5A, the GRS transcript was rapidly degraded when incubated in the cell-free system. A decay intermediate, however, was detected as five bands whose 3′ ends mapped to a 5-base region just downstream of the G-rich tract. A similar intermediate was also observed if a 20-base poly(G) tract was inserted into the SV RNA (data not shown). We believe this group of bands to be true intermediates in RNA turnover since (i) their production was temporally linked to turnover of the input RNA, (ii) the intermediates themselves were very unstable in the system, and (iii) similar intermediates were seen when several other RNAs containing a G-rich tract were incubated in the in vitro stability system (Fig. 5B; data not shown). These observations rule out a 5′-to-3′ exonuclease as being the major pathway of degradation in our system and suggest that either a 3′-to-5′ exonuclease or an endonuclease is the responsible activity.

We considered that a 3′-to-5′ exonuclease was the most likely mechanism for turnover in our system because the highly structured poly(G) tract would likely serve as a transient block to the progress of such an activity. To rule out the possibility that a G tract-specific endonuclease was involved in the formation of the observed intermediate, we blocked the 3′ end of our test transcript (SVE-G) with a 30-base poly(A) tail [SVE-G Poly(A)$^+$] and assessed the formation of the intermediate in the in vitro system. As seen in Fig. 5C, the poly(A) tail both stabilized the SVE-G transcript and blocked the formation of the transient intermediate. In addition, no degradation

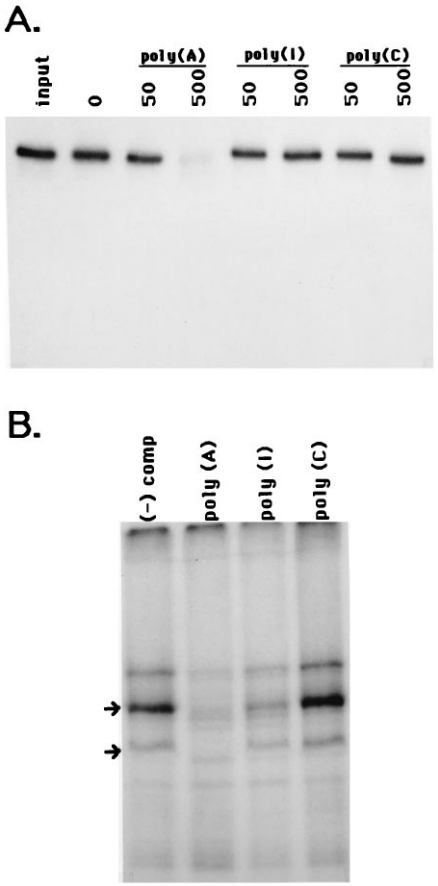

FIG. 4. Titration of poly(A) binding proteins from the poly(A) tail inhibits its ability to stabilize RNAs in vitro. (A) SVA60, a derivative of SV RNA which contains a 60-base poly(A) tail, was incubated in the in vitro RNA stability system in the presence of the indicated amounts (in nanograms) of cold homopolymer competitor RNA. Reaction products were purified and analyzed on a 5% acrylamide gel containing 7 M urea. (B) SVA60 RNA, radiolabeled at adenylate residues, was incubated in the in vitro RNA stability system in the presence of EDTA (to stabilize all transcripts) and 500 ng of the indicated homopolymer competitor RNA. After 10 min, reaction mixtures were irradiated with UV light to covalently cross-link proteins to the RNA. Following RNase treatment, cross-linked proteins containing short radiolabeled RNA oligomers were analyzed on a 10% acrylamide gel containing SDS.

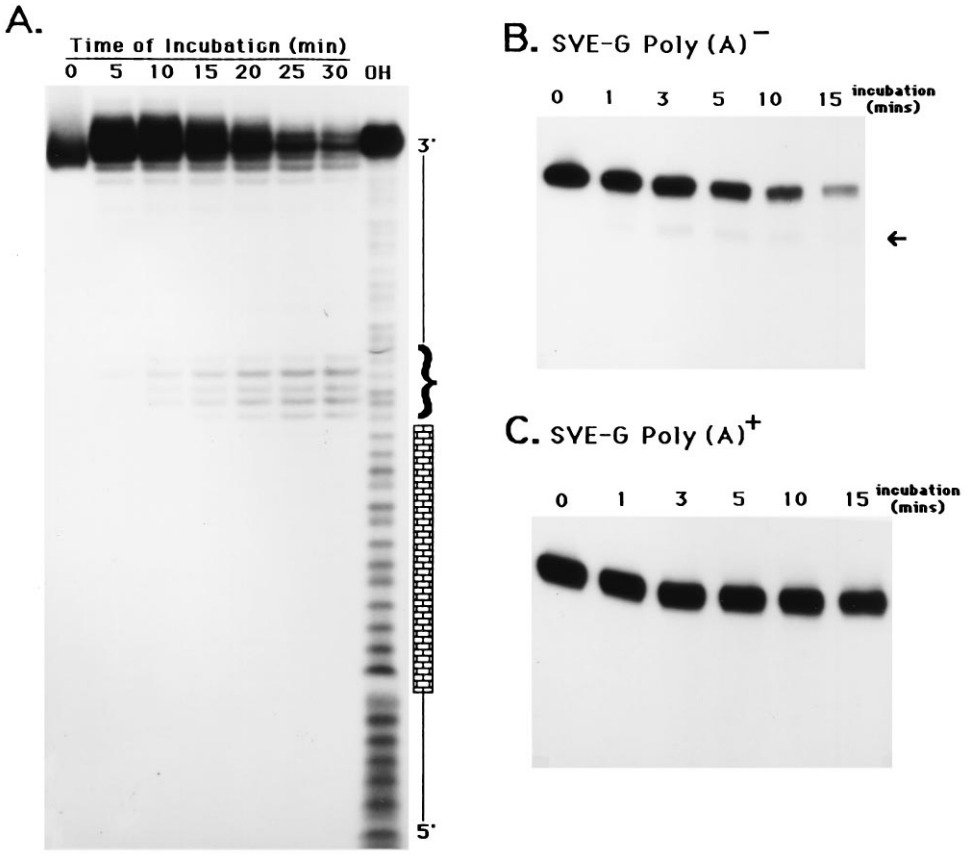

FIG. 5. A 3′-to-5′ exonuclease activity is the major pathway of RNA turnover in the in vitro RNA stability system. (A) A 51-base RNA containing a 14-base G-rich tract was radiolabeled at its 5′ end and incubated in the in vitro RNA stability system for the times indicated. Reaction products were purified and analyzed on a 5% acrylamide sequencing gel containing 7 M urea. Lane OH represents an alkaline ladder of GRS RNA fragments. The 0-min sample contains RNA prior to incubation in the system. The brace on the right side of the panel indicates the position of unstable degradation intermediates, while the brick-wall pattern indicates the position on the RNA ladder of the G-rich tract. (B) An RNA derived from the 3′ UTR of the SV40 early transcription unit which contained a 14-base G-rich tract [SVE-G Poly(A)$^-$] was incubated in the in vitro RNA stability system for the indicated times. Reaction products were purified and analyzed on a 5% acrylamide gel containing 7 M urea. The arrow indicates the position of the transient intermediate due to the presence of the G-rich tract. (C) A variant of the SVE-G RNA containing a 30-base poly(A) tail [SVE-G Poly(A)$^+$] was incubated in the in vitro RNA stability system for the indicated times. Reaction products were purified and analyzed on a 5% acrylamide gel containing 7 M urea.

intermediates were observed with RNAs containing G-rich tracts which were labeled exclusively at their 3′ ends with pCp (data not shown). Since it is unlikely that a modification at the 3′ end would significantly influence the action of a G-rich tract-specific endonuclease acting on an internal sequence, we conclude that the major pathway of RNA turnover in our in vitro system likely involves the action of a 3′-to-5′ exonuclease whose activity can be blocked by the placement of a 3′ poly(A) tail. The properties of the exonuclease activity in our system appear to be similar to the divalent cation-dependent, polysome-associated, RNasin-insensitive 3′-to-5′ exonuclease that has been suggested to play a role in in vitro turnover of histone mRNAs by Ross and colleagues (9).

The poly(A) tail blocks the assembly, but not the action, of the RNA turnover enzyme. We next used our in vitro RNA stability system to directly address several hypotheses to explain the mechanism of poly(A) tail-mediated RNA stability. Based on its role in translation initiation (31) and in inhibiting a decapping activity in *S. cerevisiae* (25), the poly(A) tail appears to functionally interact with the 5′ cap structure found on all mRNAs. Furthermore, all of the RNA substrates used in our studies contain 5′ ^{7m}G cap structures. In order to test if an interaction between the 5′ cap and 3′ poly(A) tail was required for poly(A)-mediated RNA stability in our in vitro system, we performed a competition experiment using increasing amounts of ^{7m}GpppG. As seen in Fig. 6, increasing the levels of cold cap analog to as high as 0.7 mM had no effect on poly(A) tail-mediated RNA stability of the SVA60 transcript over a 30-min period. Cap binding proteins were competed from RNA substrates at 0.2 mM cold cap analog in our system (as assayed by UV cross-linking using RNAs specifically labeled at the 5′ cap; data not shown). The range of cap analog used in these competition studies was also reported previously to be sufficient to affect cap-mediated translation in vitro (35). We conclude that a titratable interaction between the poly(A) tail and 5′ cap does not appear to play a role in determining transcript stability in our system.

We next addressed the significance of the position of the poly(A) tail with respect to its ability to mediate RNA stability in vitro. We compared the relative stabilities of RNAs which contained a 60-base poly(A) tract at the 3′ end (SVA60) and of an RNA which contained a 60-base poly(A) tract located 15 bases internal to the 3′ end (SVintA60). As seen in Fig. 7A, while an RNA containing a terminal poly(A) tail was stable, a similar RNA that contained a poly(A) tract 15 bases from the 3′ end was rapidly degraded. Terminal and internal poly(A)

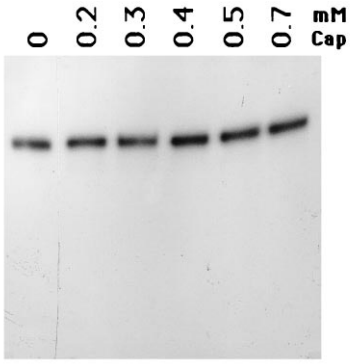

FIG. 6. A titratable interaction with the 5′ cap is not involved in poly(A) tail-mediated RNA stability. SVA60 RNA was incubated in the in vitro RNA stability system in the presence of the indicated amounts of cold ^{7m}GpppG. Reaction products were purified and analyzed on a 5% acrylamide gel containing 7 M urea.

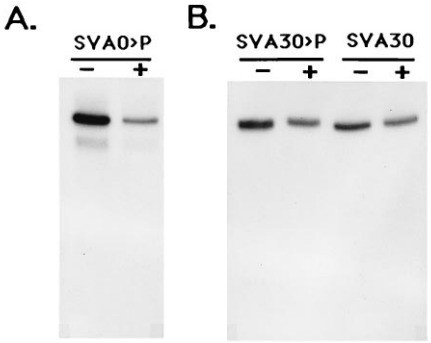

FIG. 8. Modification of the 3′ OH to a cyclic 2′,3′ phosphate does not affect RNA turnover or poly(A)-mediated RNA stability. (A) The 3′ end of the normally unstable, nonpolyadenylated SVA0 RNA was modified to a cyclic phosphate by ribozyme cleavage. The resulting RNA (SVA0>P) was incubated in the in vitro RNA stability system, and reaction products were analyzed on a 5% acrylamide gel containing 7 M urea. (B) The 3′ OH of SVA30 RNA, which contained a 30-base poly(A) tail, was modified to a cyclic phosphate (SVA30>P) by ribozyme cleavage. RNAs were incubated in the in vitro RNA stability system, and reaction products were analyzed on a 5% acrylamide gel containing 7 M urea. Lanes designated − represent input RNAs.

tracts were equally capable of interacting with poly(A) binding proteins, as indicated by UV cross-linking analysis (Fig. 7B). We conclude that the poly(A) must be located at the 3′ end of the RNA in order to function as an RNA stability element.

The positional requirements of the poly(A) tail to function in RNA stability may be due to a requirement to interact with the 3′ OH. In order to address this hypothesis, we used in vitro cleavage with synthetic hammerhead ribozymes to generate RNA transcripts each containing a terminal 2′,3′ cyclic phosphate (17). As seen in Fig. 8A, the modification of the 3′ OH to a cyclic phosphate did not affect the degradation of the normally unstable SVA0 transcript. In addition, RNAs containing a 3′ phosphate added by using pCp and RNA ligase were capable of being degraded in the system (data not shown). The presence of a 2′,3′ cyclic phosphate at the end of a 30-base poly(A) tail also did not affect the ability of the tail to effectively stabilize the SV RNA in vitro (Fig. 8B). Since the in vitro system uses relatively crude extracts, we cannot formally rule out the possibility that it contains an activity which efficiently removes the 2′,3′ cyclic phosphate from test RNAs. We tentatively conclude that the 3′ OH group of the RNA is not required for either the 3′-to-5′ exonuclease to be able to act on the transcript or the poly(A) tail to stabilize the RNA.

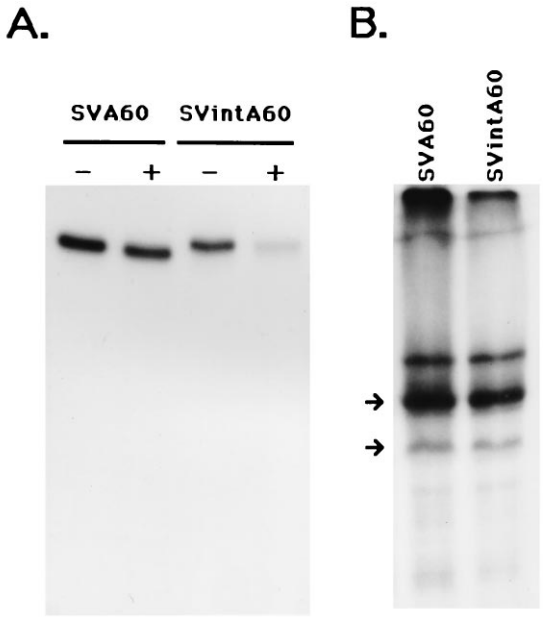

FIG. 7. Placement of the poly(A) tail-PABP complex 15 bases internal to the 3′ end inhibits its ability to stabilize RNAs in the in vitro system. (A) SVA60 RNA, or a derivative containing an additional 15 nucleotides after the poly(A) tract on its 3′ end (SVintA60), were incubated in the in vitro RNA stability system (+). Reaction products were purified and analyzed on a 5% acrylamide gel containing 7 M urea. Lanes designated − represent input RNAs. (B) SVA60 or SVintA60 RNAs, radiolabeled at adenylate residues, were incubated in the in vitro RNA stability system in the presence of EDTA for 10 min. Reaction mixtures were irradiated with UV light to covalently cross-link proteins to the RNA. Following RNase treatment, cross-linked proteins containing short radiolabeled RNA oligomers were analyzed on a 10% acrylamide gel containing SDS. The arrows indicate the positions of the two PABPs identified in Fig. 4.

An alternative hypothesis is that the terminal position of the poly(A) tail is required to prevent the initial assembly of the degradation machinery onto the 3′ end of the RNA. Since internally located poly(A) is not capable of stabilizing the transcript or generating an intermediate in the degradation process (Fig. 7A), the poly(A) tail complex must be unable to block the progress of the highly processive degradation activity. If this hypothesis is correct, then placement of a poly(A) tract at positions progressively farther from the 3′ end should define the approximate length of the sequence required to assemble the degradation machinery. We determined the influence of a 30-base poly(A) tract on RNA stability when it is located 1, 5, 11, or 21 bases from the 3′ end. As seen in Fig. 9, placement of the poly(A) tract 1 base from the 3′ end had no effect on its ability to stabilize the SV RNA. Locating the poly(A) tract 5 bases from the end allowed poly(A)-mediated RNA stability at a reduced efficiency. Internal poly(A) tracts located 11 bases from the 3′ end afforded only limited RNA stability, while poly(A) located 15 (Fig. 7) or 21 bases (Fig. 9) from the end failed to significantly stabilize the SV RNA in our in vitro system. Similar results were obtained by placing internal poly(A) tracts on the myc RNA (data not shown). Furthermore, the sequences inserted between the poly(A) sequence and the 3′ OH in the constructs used in Fig. 7 and 9 were different, arguing against a nonspecific effect due to sequence or structural artifacts. We conclude that the poly(A) tail must be located within 5 bases of the 3′ end in order to effectively function as an RNA stability element in vitro.

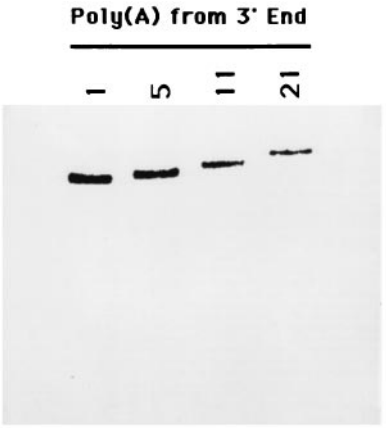

FIG. 9. Placement of the poly(A) tract at positions internal to the 3′ end of the RNA progressively reduces its ability to function as a stabilizing element. Derivatives of SVA30 which contained a 30-base poly(A) tract located 1, 5, 11, or 21 bases from the 3′ end (lanes 1, 5, 11, and 21, respectively) were incubated in the in vitro RNA stability system, and reaction products were analyzed on a 5% acrylamide gel containing 7 M urea.

DISCUSSION

We have developed an in vitro RNA stability system using standard HeLa cell extracts in which poly(A)$^-$ transcripts are rapidly and efficiently degraded by a 3′-to-5′ exonuclease. The key observation which validates this system as an in vitro model for general mRNA turnover is that the addition of a poly(A) tail of ≥30 adenylates to the 3′ end of the transcripts stabilized RNAs in the presence of available poly(A) binding proteins. Since mRNAs are rapidly degraded in vivo only when their 3′ ends are deadenylated to a poly(A) length of approximately 30 bases (30), our in vitro system faithfully reproduces several of the general aspects of mRNA turnover and should prove useful in dissecting general mechanisms involved in mRNA degradation and poly(A)-mediated RNA stability. In its current state, our system is only a partial reconstruction of regulated mRNA turnover since it lacks a deadenylation activity. Since the rate of deadenylation is directly related to the relative $t_{1/2}$ of an mRNA (4, 30), in vivo differences in relative mRNA stabilities are not reflected in this system. Our initial goals with this in vitro system are to elucidate the mechanistic aspects of the degradation machinery as well as the mechanism of poly(A)-mediated mRNA stability.

The in vitro system described here offers several advantages over established mRNA degradation in vitro systems. First, background ribonuclease degradation is minimal in our system, allowing for clean, interpretable data. Second, since it uses a well-established extract methodology, the system should be readily reproducible. Third, as a partial system, it should be useful in identifying and purifying deadenylation activities and regulatory factors in reconstitution assays. Finally, it allows the role of the poly(A) tail in mRNA stability to be assessed in the absence of confounding deadenylation activities. Our system, therefore, should provide significant biochemical insights into the processes of general and regulated mRNA stability.

Most of the substrates we have tested so far in our in vitro stability system are derived from the 3′ UTR of mRNA, a region known to contain several destabilizing and/or stabilizing elements (30). We have found no evidence, however, for a sequence requirement for a transcript to be efficiently turned over in our in vitro system. Transcripts derived from the polylinker regions of pGEM plasmids, for example (Fig. 5A), are also rapidly degraded in our system. The only consistent structural requirement we have observed for an unstable transcript is that its 3′ end must be unstructured and/or not stably associated with a protein complex. Blocking the 3′ end with a histone-derived stem-loop or a poly(A) tail, for example, results in efficient RNA stabilization. RNAs can also be stabilized by placement of a highly structured domain such as poly(G) at the 3′ end (data not shown). This observation is consistent with a model in which assembly of the degradation machinery on the 3′ end requires a generally unstructured region and in which mRNA stability is afforded by inhibiting this assembly.

All nuclear and cytoplasmic non-mRNA transcripts have their 3′ ends blocked by secondary structures and/or protein interactions, perhaps to stabilize them from the assembly of the exonuclease which is active in our in vitro system. Since 3′-end structures or protein complexes in general appear to be capable of stabilizing an RNA transcript, the question arises as to why evolution has chosen poly(A) as the 3′-end stabilization element for most mRNAs. Since RNA stability must be a regulated process to allow efficient macromolecular synthesis, the answer to this question probably lies in the fact that the cell has evolved a specific, regulatable deadenylation machinery which efficiently acts on transcripts selected for turnover. The assembly or action of this degradation machinery on the poly(A) tail is blocked only through the interaction of poly(A) binding proteins. Shortening of the tail to <30 bases results in inefficient interaction of the mRNA with these proteins and, therefore, rapid degradation of most transcripts. The levels of small stable poly(A)$^-$ RNAs which contain alternate 3′-end stabilizing elements are, therefore, much harder to regulate. Histone mRNAs, which contain 3′ stem-loops, are the exception to this, as the presence of this structure and its associated binding factors (13) allows the stability of these transcripts to be regulated in a cell cycle-specific manner (22).

A 3′-to-5′ exonuclease is the major pathway of RNA degradation in our in vitro system. It has properties very similar to those of the activity recently purified from the in vitro system of Ross and colleagues (9) which has been implicated in the turnover of histone mRNAs. First, the exonuclease present in our system is generated by a 420 mM salt wash of a nuclear pellet. This is similar to the 3′-to-5′ exonuclease identified by the Ross laboratory which is solubilized from a polysomal fraction by 0.3 to 0.5 M KCl. In addition, our 3′-to-5′ exonuclease activity requires divalent cations, is not affected by inhibitors of the RNase A family of enzymes, is stimulated by ATP, and is sensitive to proteases. Unexpectedly, the exonuclease apparently does not require a free 3′ OH to degrade RNAs in our in vitro system. Further biochemical characterization of components and substrate requirements for this exonuclease activity, as well as its regulation, will be approached by fractionation of the in vitro system.

The poly(A) tail alone cannot function as a stabilizing element without the association of *trans*-acting factors, presumably the poly(A) binding proteins. In addition to the competition data shown in Fig. 4 and reference 6, this conclusion is supported by mutation studies of the poly(A) tail through the site-specific substitution with increasing amounts of C residues. The debilitating effect of these mutations on poly(A) tail function correlates perfectly with a reduced ability of the variant tails to interact with poly(A) binding proteins, as assayed by UV cross-linking (data not shown). The observation that 30 or more consecutive adenylates are required for efficient stabilization implies that the binding of one or at most two molecules of poly(A) binding proteins, perhaps in a cooperative manner, is required for poly(A) tail function (3). The poly(A) tail, therefore, is likely to direct the assembly of a highly stable,

cooperative structural complex which functions in both mRNA stabilization and translation. This multifunctional complex is likely to interact with other proteins to accomplish its many roles in mRNA biology. Our in vitro system allows a unique opportunity to identify both the interactions of the poly(A) tail and their functional significance to the process of RNA stability.

The observation that the poly(A) tail loses its ability to stabilize the transcript when it is placed 15 bases from the 3′ end (Fig. 7) may have important implications for mechanisms of regulated mRNA stabilization. This especially applies to models which suggest that destabilizing elements such as the AU-rich element can function by displacing proteins from the poly(A) tail, thereby increasing its deadenylation rate (6). The construct containing an internal poly(A) tract may be comparable to the situation in which the terminal poly(A) binding protein is removed from a full-length poly(A) tail. Our data suggest that this situation would not merely accelerate deadenylation but would provide an assembly pad for the complex of degradation enzymes which would rapidly decay the entire transcript. Our data favor models which suggest that the deadenylation machinery is the target for activation by the destabilizing elements (2, 32). We suggest, therefore, that the function of the poly(A) tail is to prevent the assembly of a degradation complex on the transcript and that regulation of deadenylation by elements in the body of the mRNA may be through an independent, distributive poly(A)-specific nuclease. Finally, it may be surprising that the placement of the poly(A) tail internal to the 3′ end did not result in the identification of degradation intermediates. Since messenger ribonucleoprotein particles (not naked RNA) are the substrates for the degradation machinery, the degradation complex is probably designed to disrupt most RNA-protein interactions once it has assembled on an RNA substrate.

The in vitro system described in this report faithfully reproduces the processes of RNA degradation and stabilization by the poly(A) tail. It does not reproduce aspects of regulated turnover, deadenylation, or the association of RNA turnover with mRNA translation. Since we are using a fractionated cellular extract, it is possible that other aspects of RNA turnover can be reconstituted in our system through add back experiments of cytoplasmic extracts. Our system, therefore, could provide a unique assay for the purification of components involved in these other processes, just as cytoplasmic S100 extracts have proven instrumental in the identification of several regulatory splicing factors (23).

In summary, we have demonstrated that the poly(A) tail can stabilize RNAs from a major 3′-to-5′ exonuclease activity by preventing the association, but not the action, of the degradation machinery. In addition, we have developed a unique in vitro system which should prove very useful in obtaining a full understanding of the general aspects of mRNA turnover and stabilization.

ACKNOWLEDGMENTS

We thank Fan Chen and Sanjay Bhaskaran for helpful discussions.

This work was supported by funds provided by the Pew Scholars Program in the Biomedical Sciences, the New Jersey Commission on Cancer Research, and NIH grant AI31165 to J.W. L.P.F. was supported by a fellowship from the Graduate School of Biomedical Sciences.

REFERENCES

1. **Aiyar, A., and J. Leis.** 1993. Modification of the megaprimer method of PCR mutagenesis: improved amplification of the final product. BioTechniques **14:**366–369.
2. **Astrom, J., A. Astrom, and A. Virtanen.** 1992. Properties of a hela cell 3′ exonuclease specific for degrading poly(A) tails of mammalian mRNA. J. Biol. Chem. **267:**18154–18159.
3. **Baer, B. W., and R. D. Kornberg.** 1983. The protein responsible for the repeating structure of cytoplasmic poly(A)-ribonucleoprotein. J. Cell Biol. **96:**717–721.
4. **Beelman, C. A., and R. Parker.** 1995. Degradation of mRNA in eukaryotes. Cell **81:**179–183.
5. **Belgrader, P., J. Cheng, X. Zhou, L. S. Stephenson, and L. E. Maquat.** 1994. Mammalian nonsense codons can be *cis* effectors of nuclear mRNA half-life. Mol. Cell. Biol. **14:**8219–8228.
6. **Bernstein, P., S. W. Peltz, and J. Ross.** 1989. The poly(A)-poly(A) binding protein complex is a major determinant of mRNA stability in vitro. Mol. Cell. Biol. **9:**659–670.
7. **Berstein, P., and J. Ross.** 1989. Poly(A), poly(A) binding protein and the regulation of mRNA stability. Trends Biochem. Sci. **14:**373–377.
8. **Brewer, G., and J. Ross.** 1988. Poly(A) shortening and degradation of the 3′ A+U-rich sequences of human c-*myc* mRNA in a cell-free system. Mol. Cell. Biol. **8:**1697–1708.
9. **Carruccio, N., and J. Ross.** 1994. Purification of a human polyribosome-associated 3′ to 5′ exonuclease. J. Biol. Chem. **269:**31814–31821.
10. **Chen, C.-Y. A., and A.-B. Shyu.** 1995. AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem. Sci. **20:**465–470.
11. **Chen, C.-Y., A. N. Xu, and A.-B. Shyu.** 1995. mRNA decay mediated by two distinct AU-rich elements from c-*fos* and granulocyte-macrophage colony-stimulating factor transcripts: different deadenylation kinetics and uncoupling from translation. Mol. Cell. Biol. **15:**5777–5788.
12. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
13. **Dominski, Z., J. Sumerel, R. J. Hanson, and W. F. Marzluff.** 1995. The polyribosomal protein bound to the 3′ end of histone mRNA can function in histone pre-mRNA processing. RNA **1:**915–923.
14. **Furuichi, Y., A. LaFiandra, and A. J. Shatkin.** 1977. 5′-terminal structure and mRNA stability. Nature **266:**235–239.
15. **Gorlach, M., C. G. Burd, and G. Dreyfuss.** 1994. The mRNA poly(A) binding protein: localization, abundance, and RNA-binding specificity. Exp. Cell Res. **211:**400–407.
16. **Green, M. R.** 1991. Biochemical mechanisms of constitutive and regulated pre-mRNA splicing. Annu. Rev. Cell Biol. **7:**559–599.
17. **Haseloff, J., and W. L. Gerlach.** 1988. Simple RNA enzymes with new and highly specific endoribonuclease activities. Nature **334:**585–591.
18. **Huang, Y., and G. G. Carmichael.** 1996. Role of polyadenylation in nucleocytoplasmic transport of mRNA. Mol. Cell. Biol. **16:**1534–1542.
19. **Keller, W.** 1995. No end yet to messenger RNA 3′ end processing! Cell **81:**829–832.
20. **Land, H., L. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature **304:**596–602.
21. **Lieberman, A. P., P. M. Pitha, and M. L. Shin.** 1992. Poly(A) removal is the kinase-regulated step in tumor necrosis factor mRNA decay. J. Biol. Chem. **267:**2123–2126.
22. **Marzluff, W. F., and N. B. Pandey.** 1988. Multiple regulatory steps control histone mRNA concentrations. Trends Biochem. Sci. **13:**49–52.
23. **Mayeda, A., and A. Krainer.** 1992. Regulation of alternative pre-mRNA splicing by hnRNP A1 and splicing factor SF2. Cell **68:**365–375.
24. **Min, H., R. C. Chan, and D. L. Black.** 1995. The generally expressed hnRNP F is involved in a neural-specific pre-mRNA splicing event. Genes Dev. **9:**2659–2671.
25. **Muhlrad, D., C. J. Decker, and R. Parker.** 1994. Deadenylation of the unstable mRNA encoded by the yeast MFA2 gene leads to decapping followed by 5′ to 3′ digestion of the transcript. Genes Dev. **8:**855–866.
26. **Nair, A. P. K., S. Hahn, R. Banholzer, H. H. Hirsch, and C. Moroni.** 1994. Cyclosporin A inhibits growth of autocrine tumour cell lines by destabilizing interleukin-3 mRNA. Nature **369:**239–242.
27. **Peppel, K., and C. Baglioni.** 1991. Deadenylation and turnover of interferon-β mRNA. J. Biol. Chem. **266:**6663–6666.
28. **Qian, Z., and J. Wilusz.** 1991. An RNA-binding protein specifically interacts with a functionally important domain of the downstream element of the simian virus 40 late polyadenylation signal. Mol. Cell. Biol. **11:**5312–5320.
29. **Ross, J.** 1993. mRNA decay in cell-free systems, p. 417–448. *In* J. Belasco and G. Brawerman (ed.), Control of mRNA stability. Academic Press, Inc., San Diego, Calif.
30. **Ross, J.** 1995. mRNA stability in mammalian cells. Microbiol. Rev. **59:**423–450.
31. **Sachs, A.** 1990. The role of poly(A) in the translation and stability of mRNA. Curr. Opin. Cell Biol. **2:**1092–1098.
32. **Sachs, A., and J. Deardorff.** 1992. Translation initiation requires the PAB-dependent poly(A) ribonuclease in yeast. Cell **70:**961–973.
33. **Schiavi, S. C., J. G. Belasco, and M. E. Greenberg.** 1992. Regulation of proto-oncogene mRNA stability. Biochim. Biophys. Acta **1114:**95–106.
34. **Shyu, A.-B., J. G. Belasco, and M. E. Greenberg.** 1991. Two distinct destabilizing elements in the c-fos message trigger deadenylation as a first step in rapid mRNA decay. Genes Dev. **5:**221–231.
35. **Tarun, S. Z., Jr., and A. Sachs.** 1995. A common function for mRNA 5′ and

3′ ends in translation initiation in yeast. Genes Dev. **9:**2997–3007.
36. **Vreken, P., and H. A. Raue.** 1992. The rate-limiting step in yeast PGK1 mRNA degradation is an endonucleolytic cleavage in the 3′ terminal part of the coding region. Mol. Cell. Biol. **12:**2986–2996.
37. **Wahle, E.** 1992. A novel poly(A) binding protein acts as a specificity factor in the second phase of messenger RNA polyadenylation. Cell **66:**759–768.
38. **Weiss, I. M., and S. A. Liebhaber.** 1995. Erythroid cell-specific mRNA stability elements in the α2-globin 3′ nontranslated region. Mol. Cell. Biol. **15:**2457–2465.
39. **Wilusz, J., D. I. Feig, and T. Shenk.** 1988. The C proteins of heterogeneous ribonucleoprotein complexes interact with RNA sequences downstream of polyadenylation cleavage sites. Mol. Cell. Biol. **8:**4477–4483.
40. **Wilusz, J., and T. Shenk.** 1988. A 64 kDa nuclear protein binds to RNA segments that include the AAUAAA polyadenylation motif. Cell **52:**221–228.
41. **Wilusz, J., and T. Shenk.** 1990. A uridylate tract mediates efficient heterogeneous nuclear ribonucleoprotein C protein-RNA cross-linking and functionally substitutes for the downstream element of the polyadenylation signal. Mol. Cell. Biol. **10:**6397–6407.