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The Poly(A)-Poly(A)-Binding Protein Complex Is a Major Determinant of mRNA Stability In Vitro

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Using an in vitro mRNA decay system, we investigated how poly(A) and its associated poly(A)-binding protein (PABP) affect mRNA stability. Cell extracts used in the decay reactions were depleted of functional PABP either by adding excess poly(A) competitor or by passing the extracts over a poly(A)-Sepharose column. Polyadenylated mRNAs for β-globin, chloramphenicol acetyltransferase, and simian virus 40 virion proteins were degraded 3 to 10 times faster in reactions lacking PABP than in those containing excess PABP. The addition of purified *Saccharomyces cerevisiae* or human cytoplasmic PABP to PABP-depleted reactions stabilized the polyadenylated mRNAs. In contrast, the decay rates of nonpolyadenylated mRNAs were unaffected by PABP, indicating that both the poly(A) and its binding protein were required for maintaining mRNA stability. A nonspecific single-stranded binding protein from *Escherichia coli* did not restore stability to polyadenylated mRNA, and the stabilizing effect of PABP was inhibited by anti-PABP antibody. The poly(A) tract was the first mRNA segment to be degraded in PABP-depleted reactions, confirming that the poly(A)-PABP complex was protecting the 3′ region from nucleolytic attack. These results indicate that an important function of poly(A), in conjunction with its binding protein, is to protect polyadenylated mRNAs from indiscriminate destruction by cellular nucleases. A model is proposed to explain how the stability of an mRNA could be affected by the stability of its poly(A)-PABP complex.

Most newly synthesized mRNA molecules contain a poly(A) tract when they enter the cytoplasm of a nucleated cell. Therefore, poly(A) is presumed to play a significant role in mRNA synthesis, translation, and/or metabolism. However, the exact functions of poly(A) and its associated poly(A)-binding protein (PABP) are incompletely understood. Poly(A) is potentially involved in any of four stages of mRNA metabolism: splicing, nucleus-to-cytoplasm transport, translation, and stability (reviewed in references 47 and 50). Although polyadenylation and splicing are linked, they can occur independently (72), and polyadenylation is not an absolute prerequisite for transport, because some nonadenylated RNAs are transported efficiently (3, 26, 73). Some viral mRNAs are synthesized exclusively in the cytoplasm and are polyadenylated, indicating an extranuclear role for poly(A) (1, 5, 19). There is evidence for a link between poly(A) and translation, especially in lower organisms (16–18, 21, 47, 62), and some experiments suggest that poly(A) affects mRNA half-lives (see below).

The role of PABP in mRNA metabolism is also unclear, although its highly specific binding properties imply that its function is linked with at least one function of poly(A) (9, 35). The molecular weight of cytoplasmic PABP varies from 72,000 in mammals (9, 23) to 68,000 in *Saccharomyces cerevisiae* (2, 58, 59) to 31,000 in slime molds (36, 37). Yeast and human PABPs share a high degree of homology within the N-terminal region, which is responsible for the poly(A)-binding function. They diverge in the C-terminal third of the protein (23). PABP interacts with poly(A), forming an ordered, nucleosomelike complex with a periodicity of 25 to 27 nucleotides, and its affinity for poly(A) is at least 100-fold greater than that for most other polynucleotides (6, 7). Despite its high affinity for poly(A), it can exchange or migrate from one poly(A) molecule to another (59), a property which might be important in terms of its proposed function as a determinant of mRNA stability (see Discussion). Deletion of *PAB* (the PABP gene) from the yeast *S. cerevisiae* is lethal, indicating that PABP is an essential protein (59).

Some experiments with intact cells provide compelling, but indirect, evidence that poly(A) metabolism is linked with mRNA stability (reviewed in references 10, 51, 53, and 61). For example, the poly(A) tracts of some mRNAs are shortened and ultimately removed in a time-dependent manner, implying that poly(A) removal precedes degradation of the mRNA body (13, 25, 39, 52, 70). This observation, in turn, has strengthened the notion that poly(A) protects some mRNAs from rapid, indiscriminate degradation. However, direct evidence supporting a poly(A) protection function has been elusive, for several reasons. First, inhibitors that interfere with polyadenylation can affect other ATP-dependent processes and, therefore, lack complete specificity. Second, it has been difficult to exploit genetic approaches for generating full-length, deadenylated mRNAs in cells. Mutations in genomic polyadenylation signals can interfere with polyadenylation when the mutated genes are transfected into cells, but the resulting transcripts usually bear little resemblance to the wild-type mRNA. They are frequently elongated past the normal 3′ terminus and have heterogeneous 3′ ends (69).

In view of these and other limitations with experiments using intact cells, we have exploited an in vitro mRNA decay system to investigate how poly(A) and PABP affect mRNA turnover rates. An in vitro system provides some significant advantages over whole cells, because the decay of synthetic mRNAs, polyadenylated or not, can be assayed rapidly and

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quantitatively without the use of complicated pulse-chase labeling techniques. Furthermore, PABP levels can be varied systematically, permitting the functions of poly(A) and PABP to be analyzed independently. The mRNA decay system that we have described includes either polysomes or a soluble, high-salt extract of polysomes (54, 55). The mRNA-degrading enzymes are associated with the polysomes and are solubilized by incubation of the polysomes in a buffer containing 0.3 to 0.5 M KCl (55). The polysomes are then pelleted, and the supernatant or ribosomal salt wash (RSW) fraction is harvested. Both polysomes and the RSW fraction contain PABP.

Several observations indicate that this system is a valid model for studying mRNA turnover and poly(A) function. (i) The rank order of mRNA decay rates in cells is reflected in vitro. For example, c-*myc* and histone mRNAs are degraded rapidly in cells and in vitro, whereas β- and γ-globin mRNAs are stable in both (54, 56). (ii) Histone mRNAs are degraded in a 3′-to-5′ direction by one or more exonucleases, both in cells and in vitro, and the decay intermediates generated in vitro are indistinguishable from those observed in intact cells (56). Therefore, mRNA decay pathways are reproduced faithfully in vitro. (iii) The effects of poly(A) on mRNA turnover in vitro are similar to those in whole cells. For example, polyadenylated histone mRNA is at least 10-fold more stable than its deadenylated counterpart in vitro and in cells (48; reviewed in reference 38). (iv) Recent in vitro data support the observation that poly(A) removal can precede degradation of the mRNA body. The first step in the in vitro decay of c-*myc* mRNA is poly(A) removal (11). The second step is degradation of the mRNA body, and this step does not begin until most or all of the poly(A) tract is removed. In contrast, both the poly(A) and the body of a stable mRNA, γ-globin, remain intact over long incubation times. These observations suggest that poly(A) can protect mRNA from nucleolytic attack. Once the poly(A) is removed, the mRNA body can be degraded.

These results also raised three questions regarding the relationship between poly(A) and mRNA stability. (i) Is the stability of an mRNA molecule proportional to the stability of its poly(A) tract? As discussed above, an unstable mRNA had an unstable poly(A) tract in vitro, while a stable mRNA had a stable poly(A) tract (11). (ii) Does poly(A) protect the 3′ regions of mRNAs from nuclease attack, and does it thereby contribute significantly in determining the half-lives of individual mRNAs? (iii) If so, what factors account for differential poly(A) shortening among different mRNAs? How can the poly(A) tract of an unstable mRNA be degraded faster than that of a stable mRNA when all poly(A) tracts have the same primary structure, disregarding size differences? The experiments presented here were designed primarily to investigate the second question.

We find that polyadenylated mRNAs which are normally stable in vitro become unstable when PABP is removed from the reaction mixture. Adding back cytoplasmic PABP restores stability, and PABP has no effect on mRNAs lacking poly(A). These results indicate that the poly(A)-PABP complex, not poly(A) alone, protects at least some mRNAs from indiscriminate destruction. Therefore, the most important feature of mRNA turnover, namely, that different mRNAs are degraded at different rates, is dependent on both components.

MATERIALS AND METHODS

The culturing of K562 human erythroleukemia cells and the preparation of RSW and ^{32}P-labeled human H4 histone and β-globin mRNAs have been described previously (54, 55). The polyadenylated simian virus 40 (SV40) and chloramphenicol acetyltransferase (CAT) mRNAs were synthesized with SP6 RNA polymerase to transcribe cDNA clones kindly provided by Peter Good, Janet Mertz, and Judy Callis. The in vitro mRNA decay reaction conditions and methods for mRNA analysis and densitometric scanning were as previously described (55). RSW (0.3 M KCl) was used for all of the reactions described here. Each 25-μl reaction contained 4 μg of RSW protein, corresponding to approximately 7×10^6 cells. The H mapping or oligonucleotide-directed RNase H procedure was performed as described by Brewer and Ross (11). Deadenylated β-globin mRNA was prepared by incubating the ^{32}P-labeled substrate with oligo(dT) plus RNase H (11).

S. cerevisiae PABP (p68) was prepared and kindly provided by Alan Sachs and Roger Kornberg. The protein was purified by A. Sachs and R. Kornberg from an *Escherichia coli* strain expressing the yeast p68 *PAB* gene (58, 59). Where indicated, yeast or human PABP was mixed on ice with the in vitro mRNA decay reaction components. The reaction mixtures were warmed to 37°C and incubated for various times. Both the preimmune immunoglobulin and the yeast anti-PABP antibody (anti-p68) were prepared and kindly provided by David Munroe, Richard Manrow, and Allan Jacobson. Each immunoglobulin was purified by D. Munroe, R. Manrow, and A. Jacobson by ammonium sulfate precipitation and chromatography on DEAE-cellulose and carboxymethyl cellulose. Purified immunoglobulin was preincubated with PABP on ice for 30 min. The mixture was added to chilled mRNA decay reaction mixtures, which were then mixed and brought to 37°C. For protein blotting, proteins were electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide minigel (Idea Scientific Co.) and electroblotted to nitrocellulose. The blot was incubated for 30 min in a solution of 3% (wt/vol) bovine serum albumin, washed, and incubated at 4°C for 16 h with 20 μg of purified immunoglobulin. After being washed, the blot was incubated for 3 h at 25°C with a 1:250 dilution of goat anti-rabbit immunoglobulin G-peroxidase conjugate (Sigma Chemical Co.). It was then developed by incubation for 5 min at room temperature with a 1:1 mixture of hydrogen peroxide and 4-chloro-1-naphthol solution (purchased from Kirkegaard and Perry) and photographed after being washed with water.

PABP-depleted RSW was prepared at 4°C as follows. Dry poly(A)-Sepharose (100 mg; Pharmacia, Inc.) was suspended in 2 ml of buffer A (500 mM KCl, 10 mM EDTA, 10 mM Tris chloride [pH 7.5]). The material was placed in a disposable plastic column (Bio-Rad Laboratories) and washed at room temperature, first with 10 ml of buffer B (10 mM KCl, 10 mM EDTA, 10 mM Tris chloride [pH 7.5]), then with 5 ml of phosphate-buffered formamide (20 mM phosphate [pH 6.8]), and then with 5 ml of buffer B. The column was placed in a cold room. A sample of the RSW from approximately 6×10^8 cells (325 μg of protein in 0.25 ml) was dialyzed at 4°C against 500 ml of buffer B, with one buffer change. The dialyzed RSW was applied to the column, which was washed with 5 ml of buffer B and then with 5 ml of buffer A. The original flowthrough plus the two 5-ml washes were pooled and concentrated to approximately 0.25 ml by centrifugation in a Centricon tube with a 10,000-molecular-weight cutoff (Amicon Corp.). As determined by the ^{32}P-poly(A)-binding assay (60), approximately 90% of the PABP was removed from the crude RSW by this procedure. Minor differences in several high-molecular-weight (>200,000) stained proteins were observed when crude RSW was compared with PABP-

depleted RSW by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). However, as determined by the decay of the ^{32}P-mRNAs, 80 to 100% of the mRNA-degrading activity was recovered in the PABP-depleted RSW (see Results). In some experiments, the poly(A)-Sepharose column was further washed with 2 M guanidine hydrochloride–30 mM Tris (pH 7.5) in an effort to elute the bound PABP. However, active PABP was not recovered after renaturation of the eluates.

Partially purified human PABP was prepared by techniques described for yeast PABP (60). All buffers included the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 0.4 μg of leupeptin per ml, and 1.4 μg of pepstatin per ml. The starting material was polysomes from exponentially growing K562 cells. Polysomes from 3×10^9 cells (18 mg of protein) were mixed with 500 μg of poly(A) and 5 mg of poly(C) (Pharmacia) in adsorption buffer (100 mM NaCl, 30 mM Tris hydrochloride [pH 8.0]). The material was applied at 25°C to 1 g of oligo(dT)-cellulose (Collaborative Research, Inc.) in a water-jacketed column. The column was washed with 10 column volumes of high-salt buffer (330 mM NaCl, 30 mM Tris chloride [pH 8.0]), and poly(A) and bound material were eluted by washing the column with 2 column volumes of low-salt buffer (5 mM EDTA, 5 mM Tris hydrochloride [pH 8.0]) at 45°C. The low-salt eluate was chilled on ice and applied to a 2-ml cibacron blue column (Pierce Chemical Co.) in a cold room. The column was washed with 10 column volumes of PABP-binding buffer (PBB; 100 mM NaCl, 5 mM EDTA, 15 mM β-mercaptoethanol, 30 mM Tris chloride [pH 8.0]), then with 3 volumes of PBB containing 4 M NaCl rather than 100 mM NaCl, then with 3 volumes of PBB, and then with 10 volumes of PBB containing 0.5 M guanidine hydrochloride. PABP was eluted in 5 ml of buffer G (100 mM NaCl, 5 mM EDTA, 15 mM β-mercaptoethanol, 2 M guanidine hydrochloride, 30 mM Tris chloride [pH 8.0]). PABP activity was restored by diluting the eluate with PBB. These steps resulted in a purification of approximately 60-fold and a recovery of 350 μg of protein. PABP was assayed by the ^{32}P-poly(A)-binding assay (60).

E. coli single-stranded DNA-binding protein (SSB) was purchased from U.S. Biochemical Corp. The globin antisense oligodeoxynucleotide was prepared at the University of Wisconsin Biotechnology Center. Ribohomopolymers were purchased from Pharmacia, and their lengths were verified by denaturing polyacrylamide gel electrophoresis and methylene blue staining.

RESULTS

Depletion of PABP by exogenous competitor poly(A) destabilizes polyadenylated mRNA. The following observations with an in vitro mRNA decay system suggested that the poly(A)-PABP complex might protect some mRNAs from rapid destruction. (i) Deadenylated, ^{32}P-labeled mRNAs were at least 10-fold less stable than polyadenylated mRNAs (48; see also references 8, 18, 28, 29, and 33). (ii) The reactions contained sufficient excess PABP to bind all of the poly(A) on the ^{32}P-labeled mRNA substrates (P. Bernstein, unpublished data). To investigate directly whether the poly(A)-PABP complex affected mRNA stability in vitro, we asked if polyadenylated mRNAs that were normally stable became unstable in reactions depleted of PABP. Two methods were exploited to extract PABP from the RSW, which was used as the source of mRNA-degrading activity for these experiments: adding excess competitor poly(A) to the reactions or passing the RSW through a poly(A)-Sepharose column. Competitor poly(A) sequesters the PABP so that little or none is available to interact with the ^{32}P-labeled mRNAs. Poly(A)-Sepharose binds PABP with high selectivity and thereby extracts it from the RSW.

Increasing amounts of all four ribohomopolymers (each 1,000 to 2,000 nucleotides in length) were added separately to in vitro mRNA decay reactions containing RSW plus polyadenylated β-globin and nonpolyadenylated H4 histone ^{32}P-mRNAs. Reactions were incubated for 20 min at 37°C, and RNA was purified and electrophoresed. As described previously (54), β-globin mRNA was stable and histone mRNA was unstable in reactions without ribohomopolymers (Fig. 1A, lane 3). As little as 6 to 30 ng of competitor poly(A) induced destabilization of the β-globin mRNA but did not affect histone mRNA decay (Fig. 1A, lanes 4 to 6). None of the other ribohomopolymers had a comparable effect on the polyadenylated mRNA (Fig. 1A, lanes 7 to 15). Poly(G) at 150 ng per reaction destabilized it to some extent in this experiment, but the effect was too small to observe consistently (Fig. 1B). Kinetic analysis confirmed that poly(A) affected the polyadenylated substrate specifically (Fig. 1B, compare lanes 2 to 7 and 8 to 13). As estimated by scanning densitometry, it was degraded seven times faster with poly(A) than with poly(G) or no homopolymer.

Two classes of globin mRNA decay intermediates were observed in reactions with competitor poly(A). One was heterogeneous and migrated between the full-length mRNA and the deadenylated mRNA (Fig. 1B, lanes 3 to 7). We show later that this class corresponded to mRNA molecules whose poly(A) tracts were being shortened (see Fig. 9). The second intermediate, observed most clearly in Fig. 1B, lanes 6 and 7, migrated as a more discrete band at the same position as deadenylated mRNA [A(−)β]. Still smaller products were either not observed or were scarce, suggesting that the deadenylated product resulted from a holdup point in the decay process. These intermediates suggest a stepwise decay pathway in which the poly(A) is first shortened and then removed and, after a brief holdup, the deadenylated mRNA is then completely destroyed. We do not know the reason for the holdup.

Yeast cytoplasmic PABP overcomes the destabilizing effect of competitor poly(A). Since poly(C), poly(G), and poly(U) had little or no effect on the decay of the polyadenylated mRNA, it seemed likely that competitor poly(A) was sequestering the PABP in the RSW. As a result, insufficient PABP was available to bind to the ^{32}P-mRNA, whose protein-free poly(A) tract was then destroyed, permitting the mRNA body to be degraded soon thereafter. Therefore, we asked if exogenous PABP could restabilize the mRNA in the presence of competitor poly(A). Cytoplasmic PABP was purified from an *E. coli* strain expressing the yeast PABP gene (*PAB*) and was kindly provided by Alan Sachs and Roger Kornberg (58, 59). As described above, the polyadenylated mRNA was stable without competitor poly(A) but was unstable with it (Fig. 2A, compare lanes 2 and 3). Yeast cytoplasmic PABP (p68) stabilized the mRNA, even in the presence of competitor poly(A) (Fig. 2A, lanes 4 to 7). Maximal stabilization was reached with approximately 2 μg of PABP per reaction but was evident with 0.5 μg. Since each yeast PABP molecule binds 25 adenylate residues (59), each mole of homopolymer (average length, 1,000 to 2,000 residues) should bind at least 40 mol of PABP. Therefore, PABP at 2 μg per reaction represented a maximum two- to threefold molar excess over the theoretical number of PABP-binding sites available in 150 ng of competitor poly(A). mRNA

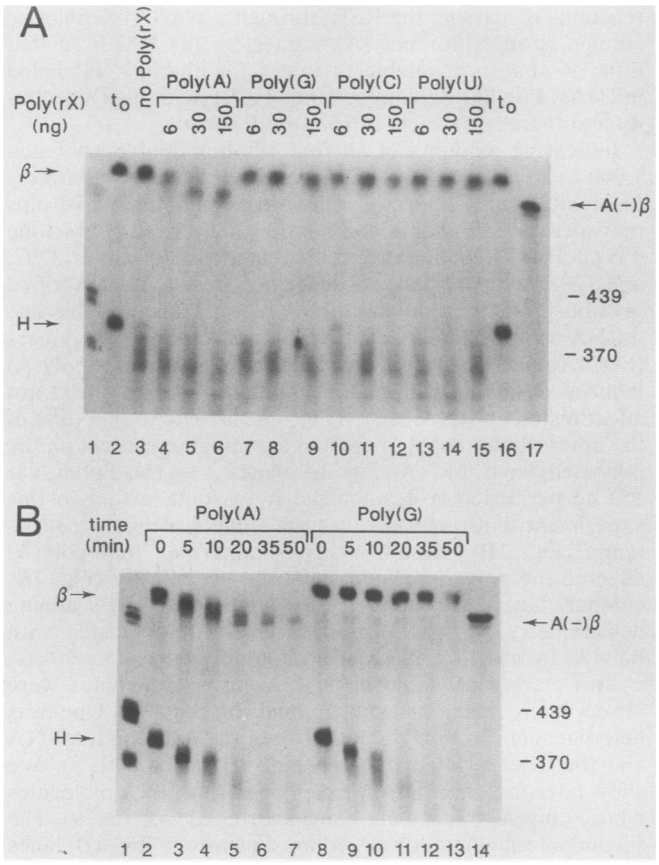

FIG. 1. Effect of competitor poly(A) on the stability of β-globin and histone ^{32}P-mRNAs. In vitro mRNA decay reactions (25-μl volumes) containing 0.3 M KCl RSW, ^{32}P-labeled human H4 histone (nonpolyadenylated), and ^{32}P-labeled and β-globin (polyadenylated) mRNAs and the indicated amounts of each ribohomopolymer were incubated for 0 or 20 min at 37°C. RNAs were extracted and electrophoresed in 4% polyacrylamide–7 M urea gels. (A) Comparison of the effects of four ribohomopolymers. Lane 1: Marker pBR322 DNA cleaved with *Hae*II and labeled with polynucleotide kinase; the sizes in nucleotides of some of the fragments are noted on the right. Lanes 2 and 16: Unincubated reactions (t_o); the locations of undegraded β-globin and histone mRNAs are indicated by β and H, respectively. Lane 3: Reaction incubated for 20 min at 37°C with no ribohomopolymer. Lanes 4 to 15: Reactions incubated for 20 min at 37°C with various amounts (nanograms per reaction) of ribohomopolymer. Lane 17: Deadenylated β-globin mRNA marker [A(−)β], prepared by incubating polyadenylated β-globin mRNA with oligo(dT) and RNase H (see Materials and Methods). (B) Time course of mRNA degradation in reactions with poly(A) or poly(G). mRNA decay reactions containing 150 ng of poly(A) or poly(G) were incubated for the indicated times, and ^{32}P-RNAs were electrophoresed as in panel A. Lane 1: DNA marker, as in panel A. Lanes 2 to 13: Reactions containing poly(A) or poly(G) incubated for the indicated times (minutes). Lane 14: Deadenylated β-globin mRNA marker, as in panel A. β and H are as in panel A.

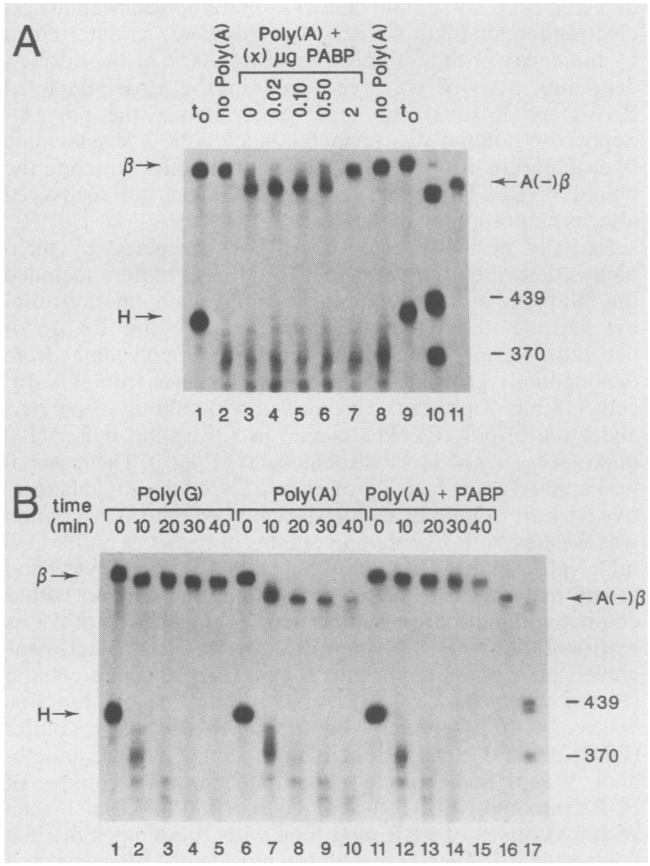

FIG. 2. Effect of purified yeast cytoplasmic PABP on the stability of β-globin mRNA. mRNA decay reactions contained polyadenylated β-globin and nonpolyadenylated H4 histone ^{32}P-mRNA substrates. In vitro mRNA decay reactions were incubated for the indicated times with or without 150 ng of competitor poly(A) and, where noted, with increasing amounts of purified, *E. coli*-derived yeast cytoplasmic PABP (59). RNAs were extracted and analyzed by electrophoresis in 4% polyacrylamide–7 M urea gels. (A) Effect of increasing amounts of PABP. Lanes 1 and 9: Unincubated reactions (t_o); undegraded β-globin and H4 histone mRNAs are indicated by β and H, respectively. Lanes 2 and 8: Reactions incubated for 20 min without poly(A) and without PABP. Lanes 3 to 7: Reactions containing 150 ng of competitor poly(A) and the indicated amounts (micrograms) of PABP. Lane 10: Marker pBR322 DNA cleaved with *Hae*II; fragment sizes in nucleotides are noted on the right. Lane 11: Deadenylated β-globin mRNA marker [A(−)β], as in Fig. 1. (B) Time course. Lanes 1 to 5: Reactions containing 150 ng of poly(G) and 2 μg of bovine serum albumin. Lanes 6 to 10: Reactions containing 150 ng of poly(A) and 2 μg of bovine serum albumin. Lanes 11 to 15: Reactions containing 150 ng of poly(A) and 2 μg of yeast PABP. Incubation times (minutes) for lanes 1 to 15 are noted at the top. Lane 16: Deadenylated β-globin mRNA marker, as in panel A. Lane 17: DNA marker, as in panel A. β and H are as in panel A.

stability was determined by the ratio of poly(A) to PABP, because fivefold more PABP was required to stabilize the mRNA when fivefold more competitor poly(A) was added (data not shown). Kinetic analysis confirmed that exogenous PABP restored the stability of the polyadenylated ^{32}P-mRNA (Fig. 2B), which was degraded six times faster without PABP (lanes 6 to 10) than with it (lanes 11 to 15).

Depleting the RSW of the endogenous PABP destabilizes polyadenylated mRNA. The RSW was chromatographed over a poly(A)-Sepharose column to deplete it of PABP. As determined by a ^{32}P-poly(A)-binding assay (60), the chromatographed RSW contained approximately 10-fold less PABP than did the native RSW. Polyadenylated β-globin and nonpolyadenylated histone mRNAs were both unstable in reactions containing PABP-depleted RSW (Fig. 3A, lane 4). Therefore, PABP depletion destabilized the polyadenylated mRNA but did not inactivate messenger RNase activity. When purified yeast cytoplasmic PABP was added to the

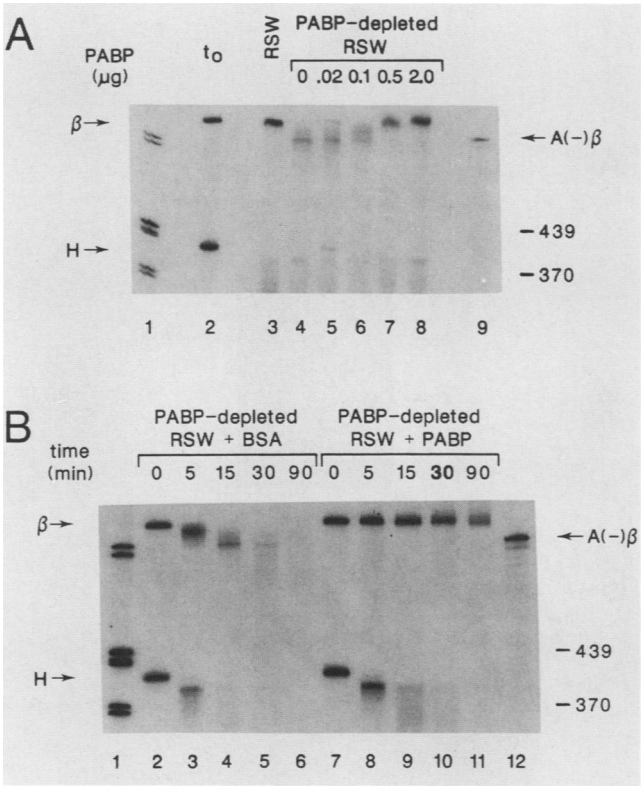

FIG. 3. Stabilization of β-globin mRNA by the addition of yeast cytoplasmic PABP to PABP-depleted RSW. The RSW was chromatographed over poly(A)-Sepharose to extract PABP (see Materials and Methods). PABP-depleted RSW was incubated under standard reaction conditions with β-globin and H4 histone ^{32}P-mRNAs and with or without purified yeast cytoplasmic PABP. RNAs were extracted and electrophoresed in 4% polyacrylamide–7 M urea gels. (A) Effect of PABP concentration on mRNA stability. Reactions were incubated for 20 min at 37°C, unless otherwise noted. Lane 1: Marker pBR322 DNA cleaved with *Hae*II; fragments sizes in nucleotides are noted on the right. Lane 2: Unincubated reaction (t_o); undegraded β-globin and H4 histone mRNAs are indicated by β and H, respectively. Lane 3: Control reaction with PABP-containing RSW that had not been chromatographed over poly(A)-Sepharose. Lane 4: Reaction with PABP-depleted RSW and 2 μg of bovine serum albumin and without PABP. Lanes 5 to 8: Reactions with PABP-depleted RSW plus increasing amounts (micrograms per reaction) of purified yeast PABP; all reactions contained a total of 2 μg of added protein, consisting of PABP and bovine serum albumin, when necessary. Lane 9: Deadenylated β-globin mRNA [A(−)β], as in Fig. 1. (B) Time course of effect of PABP on mRNA incubated with PABP-depleted RSW. Lane 1: DNA marker, as in panel A. Lanes 2 to 6: PABP-depleted RSW plus 2 μg of bovine serum albumin (BSA). Lanes 7 to 11: PABP-depleted RSW plus 2 μg of yeast PABP. Incubation times (minutes at 37°C) for lanes 2 to 11 are noted at the top. Lane 12: Deadenylated β-globin mRNA marker, as in panel A. β and H are as in panel A.

depleted RSW, the polyadenylated mRNA became stable (Fig. 3A, lanes 5 to 8), confirming that the mRNA had become unstable because the PABP had been removed. Kinetic analysis indicated that PABP stabilized the polyadenylated substrate approximately 20-fold (Fig. 3B). It is significant that histone mRNA decay rates were similar with untreated RSW and with PABP-depleted RSW supplemented with yeast PABP (Fig. 3B). This result indicates that the depletion step did not significantly modify the mRNA-degrading enzymes or other cofactors besides PABP.

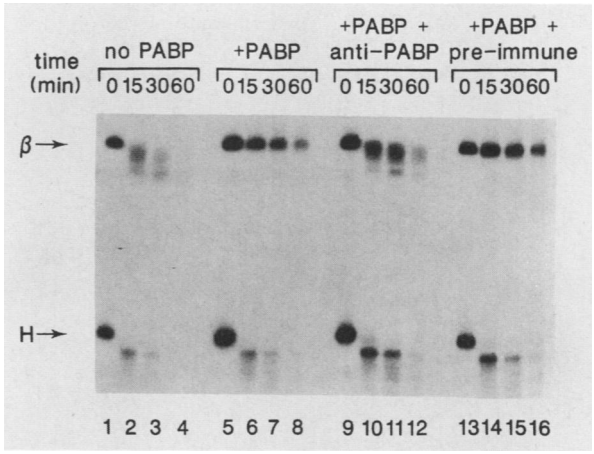

FIG. 4. Blockage by anti-p68 of stabilization of β-globin mRNA by yeast cytoplasmic PABP. All reaction mixtures contained PABP-depleted RSW. Yeast cytoplasmic PABP or bovine serum albumin was preincubated with purified anti-p68 or preimmune immunoglobulin on ice for 30 min before the other mRNA decay reaction components were added. Incubation times (minutes) at 37°C are noted at the top. RNAs were extracted and electrophoresed in 4% polyacrylamide–7 M urea gels. β and H indicate the positions of undegraded β-globin and H4 histone ^{32}P-mRNAs, respectively. Lanes 1 to 4: 10.5 μg of bovine serum albumin. Lanes 5 to 8: 10 μg of bovine serum albumin plus 500 ng of yeast PABP. Lanes 9 to 12: 10 μg of anti-p68 plus 500 ng of PABP. Lanes 13 to 16: 10 μg of preimmune immunoglobulin plus 500 ng of PABP.

Antibody to yeast PABP blocks its ability to stabilize polyadenylated mRNA. To confirm that the exogenous PABP was actually responsible for restabilizing the mRNA, we attempted to block formation of the poly(A)-PABP complex by preincubating yeast cytoplasmic PABP (p68) with purified anti-p68. Highly specific anti-p68 (see Fig. 5) was raised in rabbits with purified yeast PABP from Alan Sachs and Roger Kornberg (58, 59) as the immunogen. The antibody was prepared, purified, and kindly provided by David Munroe, Richard Manrow, and Allan Jacobson. PABP was preincubated with bovine serum albumin, preimmune immunoglobulin, or anti-p68. The mixtures were added to reactions containing PABP-depleted RSW and ^{32}P-mRNAs, the reactions were incubated at 37°C, and the RNAs were analyzed by gel electrophoresis. The polyadenylated substrate was unstable without PABP but was stabilized by it (Fig. 4, compare lanes 1 to 4 and 5 to 8). Anti-p68, but not preimmune immunoglobulin, interfered with the stabilization effect (Fig. 4, lanes 9 to 16). Antibody neutralization was not complete (Fig. 4, compare lanes 9 to 12 and 1 to 4), probably because the antibody was unable to react with all of the PABP in the reaction. Both anti-p68 and preimmune immunoglobulin slowed histone mRNA degradation slightly, but they did so to approximately the same extent. Neither had any detectable nuclease activity in reactions lacking RSW (data not shown).

To assess the specificity of the antibody and its capacity to cross-react with RSW proteins, we electrophoresed and blotted control RSW or RSW supplemented with yeast PABP and reacted the blot with anti-p68 or preimmune immunoglobulin. A prominent band was observed in the lanes containing yeast PABP but not in the lanes containing RSW alone (Fig. 5). Therefore, anti-p68 reacted specifically with yeast cytoplasmic PABP and not with components in the RSW.

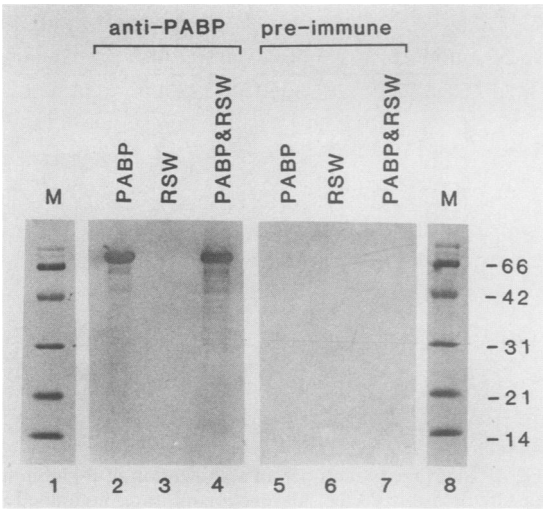

FIG. 5. Western blotting (immunoblotting) analysis of RSW and RSW supplemented with yeast PABP. Proteins were electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel and were blotted as described in Materials and Methods. The blots were incubated with either purified anti-p68 or purified preimmune immunoglobulin, as noted at the top. Antigen-antibody complexes were visualized by peroxidase staining. Lanes 1 and 8: Marker proteins; molecular weights (in thousands) are indicated on the right. Lanes 2 and 5: 2 μg of yeast cytoplasmic PABP (p68). Lanes 3 and 6: 15 μg of PABP-depleted RSW plus 2 μg of bovine serum albumin. Lanes 4 and 7: 15 μg of PABP-depleted RSW plus 2 μg of yeast cytoplasmic PABP.

Partially purified human PABP protects polyadenylated mRNA from rapid degradation. Human PABP was partially purified by the method of Sachs and Kornberg (60). The steps (Fig. 6A legend and Materials and Methods) included chromatography on oligo(dT)-cellulose and cibacron blue columns. PABP was the major protein eluted from cibacron blue (Fig. 6A), but overloading of gels revealed several additional minor bands. The preparation was estimated to be 90% pure. β-Globin mRNA was degraded at least four times more slowly in the presence than in the absence of human PABP (Fig. 6B). In this experiment and others as well, the extent of protection was similar with the human and yeast PABPs.

Poly(A) and PABP affect mRNA stability via specific interactions. To confirm that PABP stabilized the polyadenylated mRNA by binding to the 3′-terminal poly(A), we asked if PABP would stabilize deadenylated globin mRNA in reactions with PABP-depleted RSW. The polyadenylated mRNA was stabilized by PABP (Fig. 7, compare lanes 2 to 4 and 5 to 7), but the deadenylated substrate, which was identical to the polyadenylated mRNA except for the missing poly(A) tract, was degraded rapidly, and its decay was unaffected by PABP (lanes 8 to 13). Furthermore, the deadenylated β-globin mRNA had the same half-life in reactions containing or lacking exogenous poly(A) (data not shown). Thus, the normally large difference in the turnover rates of histone and globin mRNAs was not maintained when either poly(A) or PABP was missing. Moreover, the effect of PABP on mRNA decay was dependent on the presence of a poly(A) tract.

The apparent requirement for both poly(A) and PABP to maintain stability implied that unprotected mRNA was first attacked at its 3′ end, that is, at its poly(A) tract. To determine the pathway of mRNA decay under PABP-deficient conditions, we exploited an oligonucleotide-directed

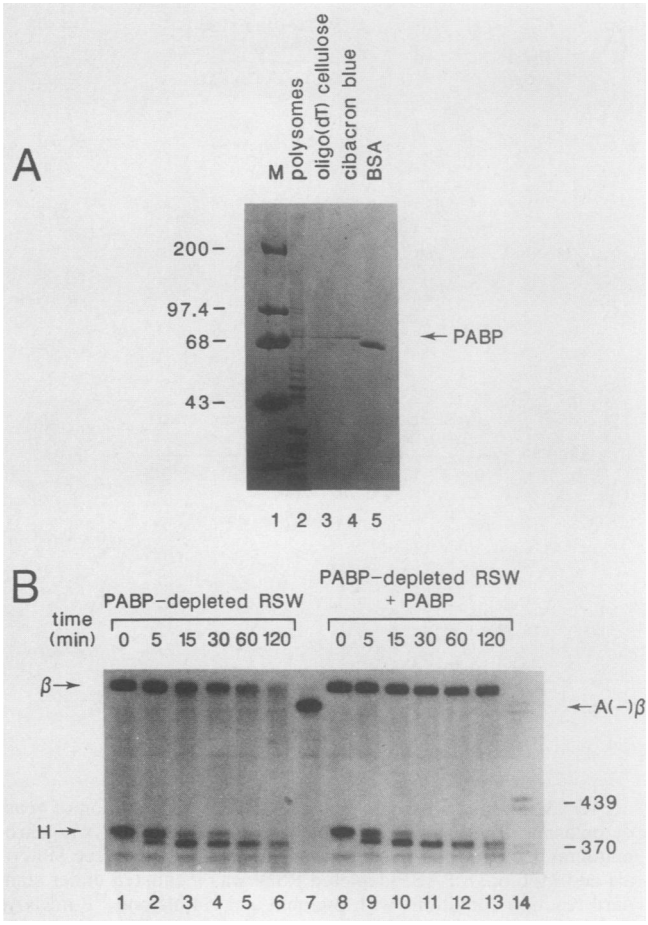

FIG. 6. Stabilization of polyadenylated mRNA by partially purified human PABP in PABP-depleted RSW. (A) Purification of PABP. Human PABP was partially purified from K562 cell polysomes (see Materials and Methods). One percent of the material from each purification step was electrophoresed in an 8% sodium dodecyl sulfate-polyacrylamide gel, which was stained with Coomassie blue. The arrow on the right indicates the PABP band. Lane 1: Molecular weight markers; sizes (in thousands) are indicated on the left. Lane 2: Starting material (proteins from K562 cell polysomes). Lane 3: Material eluted in low-salt buffer from oligo(dT)-cellulose. Lane 4: Material eluted from cibacron blue. Lane 5: Bovine serum albumin (BSA) marker. (B) mRNA stabilization by human PABP. In vitro mRNA decay reactions contained β-globin and H4 histone ^{32}P-mRNAs plus PABP-depleted RSW and were incubated for the indicated times (minutes) at 37°C. RNAs were electrophoresed in 3% polyacrylamide–7 M urea gels. The locations of polyadenylated (β) and deadenylated [A(−)β] globin mRNAs and H4 histone (H) mRNA are noted. Lanes 1 to 6: 2.5 μg of bovine serum albumin added to each reaction. Lane 7: Deadenylated β-globin mRNA, as in Fig. 1. Lane 8 to 13: 2.5 μg of partially purified human PABP (cibacron blue step) added to each reaction. Lane 14: pBR322 DNA *Hae*II marker; fragment sizes in nucleotides are noted on the right.

RNase H cleavage method (H mapping) that had been useful for analyzing the early steps in c-*myc* mRNA decay (11) (Fig. 8). Polyadenylated β-globin ^{32}P-mRNA was incubated in reactions containing nondepleted RSW plus 150 ng of either poly(A) or poly(G). Total RNA was extracted and annealed with a single-stranded oligodeoxynucleotide (25-mer) complementary to a region located between 106 and 130 nucleotides 5′ of the globin mRNA poly(A) addition site (Fig. 8).

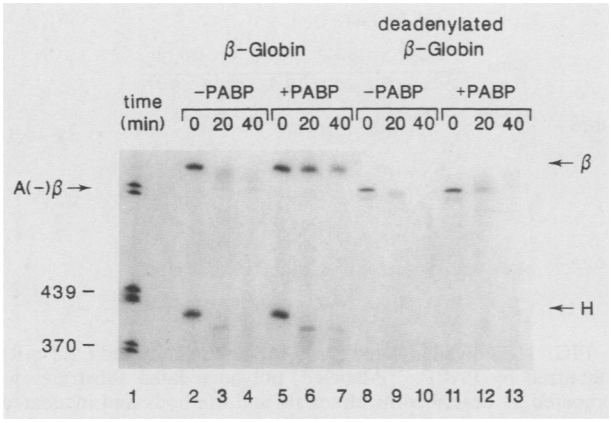

FIG. 7. Effect of yeast cytoplasmic PABP on the decay of polyadenylated versus nonpolyadenylated β-globin mRNA. In vitro mRNA decay reactions contained PABP-depleted RSW, H4 histone (H) ^{32}P-mRNA, and either polyadenylated (β) or nonpolyadenylated [A(−)β] β-globin ^{32}P-mRNA. Where indicated, 2 μg of yeast PABP was added. Reactions were incubated for the indicated times (minutes), and RNAs were extracted and electrophoresed in 4% polyacrylamide–7 M urea gels. Lane 1: Marker pBR322 DNA cleaved with *Hae*II; fragment sizes in nucleotides are noted on the left. Lanes 2 to 7: Polyadenylated β-globin mRNA (β). Lanes 8 to 13: Deadenylated β-globin mRNA [A(−)β].

The DNA-RNA hybrids were treated with RNase H, which generated two fragments, corresponding to 5′ and 3′ mRNA segments. If degradation were occurring 3′ to 5′, beginning in the poly(A) tract, the smaller 3′ fragment should have been degraded sooner than the larger 5′ one. Undegraded mRNA generated the two expected fragments (Fig. 9A, lanes 1 and 13). The size of the smaller fragment, labeled 3′-β, corresponded to the 3′-terminal 106 to 120 nucleotides of the mRNA plus the 80-nucleotide poly(A) tract. The difference between the polyadenylated and deadenylated 3′-β fragments is shown in Fig. 9A, lanes 13 and 14. The 3′ fragment was less stable in reactions with poly(A) than in those with poly(G) (Fig. 9A). More importantly, the 3′ fragment was destroyed faster than the 5′ fragment (Fig. 9B). Therefore, poly(A) was the first part of the mRNA to be degraded in PABP-depleted reactions, confirming that PABP

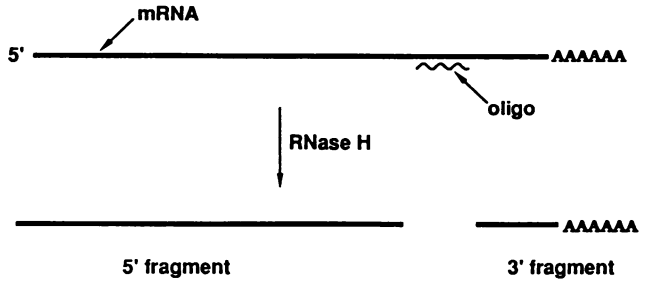

FIG. 8. RNase H mapping method for analyzing poly(A) shortening and for determining the directionality of mRNA decay. Details of the method have been described previously (11). Briefly, a synthetic antisense oligodeoxyribonucleotide is annealed to the ^{32}P-mRNA, and the hybrid is treated with RNase H, generating 5′ and 3′ mRNA fragments. The fragments are then electrophoresed. Since site-specific RNase H cleavage defines the 5′-terminal region of the 3′ fragment, small changes in the size of that fragment that occur during the in vitro incubation must result from poly(A) shortening.

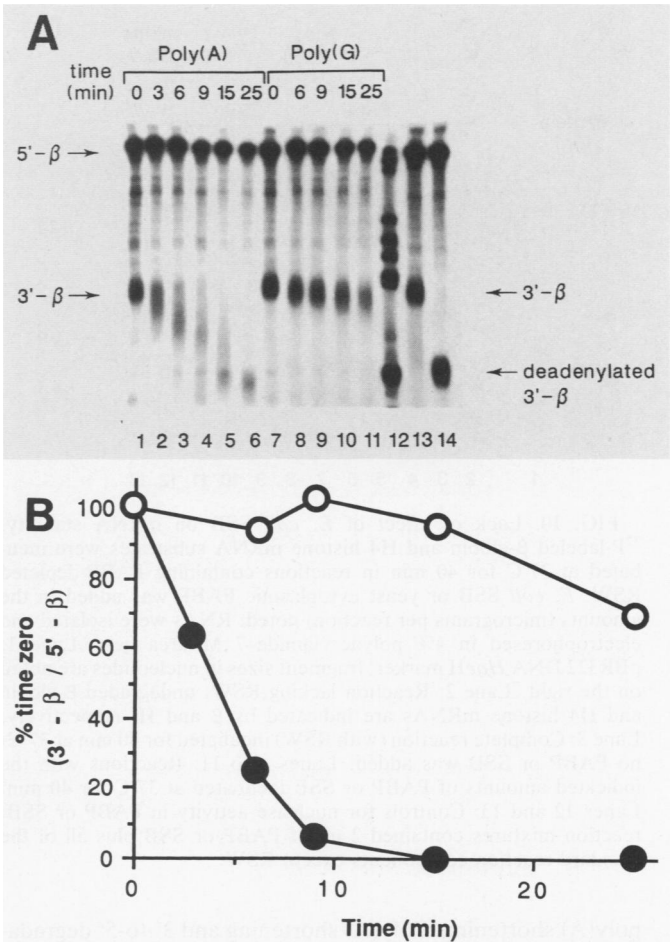

FIG. 9. Accelerated poly(A) shortening and 3′-to-5′ degradation in reactions containing competitor poly(A). (A) ^{32}P-labeled, polyadenylated β-globin mRNA was incubated for the indicated times in reactions containing RSW and 150 ng of either poly(A) or poly(G). RNA was extracted and hybridized with a 25-nucleotide single-stranded deoxynucleotide complementary to a region located 106 to 130 nucleotides 5′ of the β-globin poly(A) addition site. RNase H treatment was performed as previously described (11), and the ^{32}P-RNA fragments were electrophoresed in 8% polyacrylamide–7 M urea gels. 5′-β and 3′-β refer to the two RNase H-generated fragments (Fig. 8). Deadenylated 3′-β fragment was made by annealing the ^{32}P-mRNA with both the antisense oligonucleotide and oligo(dT) prior to RNase H digestion. Lanes 1 to 11: Reactions incubated for the indicated times (minutes). Lane 12: pBR322 DNA *Hae*III marker; the two fragments migrating at approximately the same position as the deadenylated 3′-β fragment contained 123 and 124 nucleotides; the fragments migrating slightly more slowly than the 3′-β fragment contained 184 and 192 nucleotides. Lane 13: Unincubated reaction. Lane 14: Unincubated reaction; RNA was annealed with both the antisense oligonucleotide and oligo(dT) before RNase H digestion. (B) Bands corresponding to the 5′ and 3′ fragments were scanned with a soft-laser densitometer to determine relative amounts. The ratios of undegraded fragments were plotted for reactions containing poly(A) (●) or poly(G) (○).

protected the 3′ region from nuclease attack. Intermediate-sized mRNA molecules generated during the first 9 min migrated between the undegraded 3′ fragment [with its poly(A) tract] and the deadenylated 3′ fragment. Since the 5′ ends of the 3′ fragments were determined by oligonucleotide-directed RNase H cleavage, the intermediate-sized RNAs must have corresponded to molecules undergoing

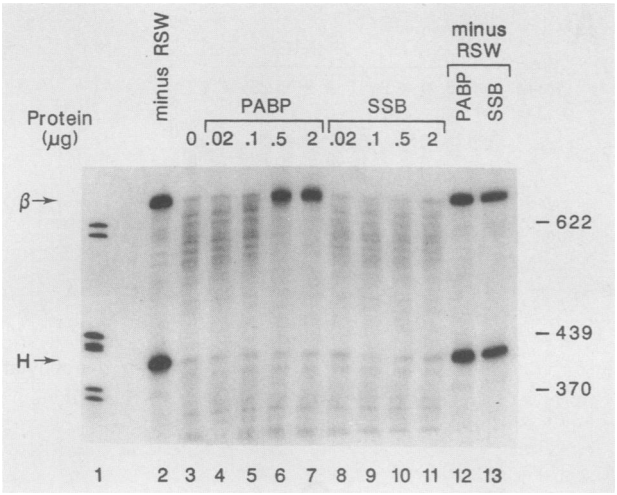

FIG. 10. Lack of effect of *E. coli* SSB on mRNA stability. ^{32}P-labeled β-globin and H4 histone mRNA substrates were incubated at 37°C for 40 min in reactions containing PABP-depleted RSW. *E. coli* SSB or yeast cytoplasmic PABP was added in the amounts (micrograms per reaction) noted. RNAs were isolated and electrophoresed in 4% polyacrylamide–7 M urea gels. Lane 1: pBR322 DNA *Hae*II marker; fragment sizes in nucleotides are noted on the right. Lane 2: Reaction lacking RSW; undegraded β-globin and H4 histone mRNAs are indicated by β and H, respectively. Lane 3: Complete reaction (with RSW) incubated for 40 min at 37°C; no PABP or SSB was added. Lanes 4 to 11: Reactions with the indicated amounts of PABP or SSB incubated at 37°C for 40 min. Lanes 12 and 13: Controls for nuclease activity in PABP or SSB; reaction mixtures contained 2 μg of PABP or SSB plus all of the standard reaction components except RSW.

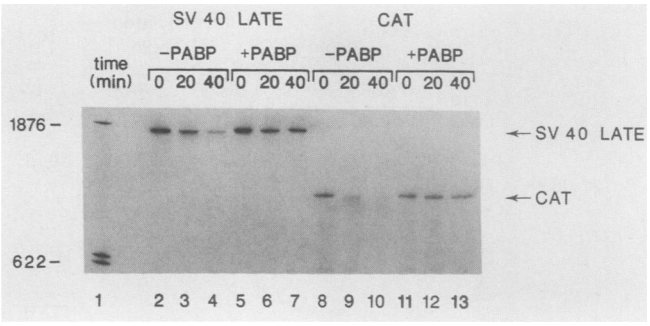

FIG. 11. Polyadenylated SV40 16S late mRNA and CAT mRNA stabilized by PABP. ^{32}P-labeled, polyadenylated substrates were prepared as described in Materials and Methods and incubated in reactions with PABP-depleted RSW. Where indicated, 2 μg of yeast PABP was added. After incubation for the indicated times (minutes) at 37°C, RNAs were extracted and analyzed by electrophoresis in 3% polyacrylamide–7 M urea gels. The positions of the undegraded mRNAs are noted on the right. Lane 1: pBR322 *Hae*II DNA marker; fragment sizes in nucleotides are noted on the left. Lanes 2 to 7: SV40 16S late mRNA. Lanes 8 to 13: CAT mRNA.

poly(A) shortening. Poly(A) shortening and 3′-to-5′ degradation were also observed in reactions with PABP-depleted RSW (data not shown), indicating that the results shown in Fig. 9 were not generated by some artifact related to competitor poly(A).

To determine if any RNA-binding protein would stabilize polyadenylated mRNA, we incubated reactions containing PABP-depleted RSW with either yeast cytoplasmic PABP or *E. coli* SSB. PABP, but not SSB, stabilized the mRNA (Fig. 10). The quantity of SSB in these reactions was sufficient to retard 100% of the mRNA in a gel migration assay (49; P. Bernstein and S. W. Peltz, unpublished observations). Therefore, although SSB could bind to the mRNA, it did not protect it from rapid degradation. Bovine serum albumin also failed to affect mRNA decay (Fig. 3 and data not shown).

To determine if the poly(A)-PABP complex protected other polyadenylated substrates, we tested CAT and SV40 16S late ^{32}P-mRNAs in vitro with PABP-depleted RSW. Both mRNAs are polyadenylated, and their half-lives are 2 h or more in cells (4, 27). Each was degraded more slowly in the presence than in the absence of PABP (Fig. 11). As estimated by scanning densitometry of the full-length mRNA bands, PABP retarded decay by at least 3- and 10-fold for the SV40 and CAT mRNAs, respectively.

DISCUSSION

These results with an in vitro mRNA decay system support the idea that poly(A) is an important component in determining mRNA stability. It protects some cellular and viral mRNAs from indiscriminate destruction and does so by virtue of its interaction with PABP. For these experiments, we chose to analyze a normally very stable mRNA, β-globin, to evaluate the extent to which the poly(A)-PABP complex affected mRNA stability. The major finding was that, when this mRNA lacks the complex, its turnover rate is drastically altered and becomes similar to that of another mRNA which is normally unstable. In other words, large differences in mRNA turnover rates are nullified when the complex is disrupted. Therefore, the complex probably plays an important role in determining the turnover rates of individual mRNAs. Our results do not suggest that all poly(A)-deficient mRNAs are necessarily very unstable. Mammalian cells contain significant quantities of functional mRNA molecules that, because they do not bind to poly(A) affinity substrates, are thought to be either deficient in or completely lacking poly(A) (22, 31, 32, 66; reviewed in reference 46). Some of these mRNAs seem to have relatively long half-lives, a fact consistent with our finding that different poly(A)-deficient mRNAs are degraded at slightly different rates in vitro (48).

Three in vitro observations suggest that the rate of poly(A) shortening can be a major factor in determining mRNA half-lives. (i) The stability of γ-globin and c-*myc* mRNAs correlates with their poly(A)-shortening rates (11). c-*myc* poly(A) is shortened rapidly, and the mRNA is unstable. γ-Globin poly(A) is shortened slowly, and the mRNA is stable. (ii) Poly(A) removal precedes degradation of the c-*myc* mRNA body (11). This result suggests, but does not prove, that poly(A) must be removed before internal sequences become susceptible to nuclease attack. (iii) A stable mRNA becomes unstable when its poly(A) tract is removed (28, 29, 48; Fig. 7). If the rate of poly(A) shortening determines the nuclease susceptibility of some mRNAs, what accounts for differences in poly(A)-shortening rates between stable and unstable mRNAs? Why should one poly(A) tract be degraded faster than another, when their primary structures are identical, disregarding size differences? Based on our observation that PABP retards poly(A) shortening (Fig. 9), we suggest that any model to account for differential poly(A)-shortening rates should probably invoke disruption of the poly(A)-PABP complex as an essential step. In other words, it might be necessary for PABP to dissociate from the

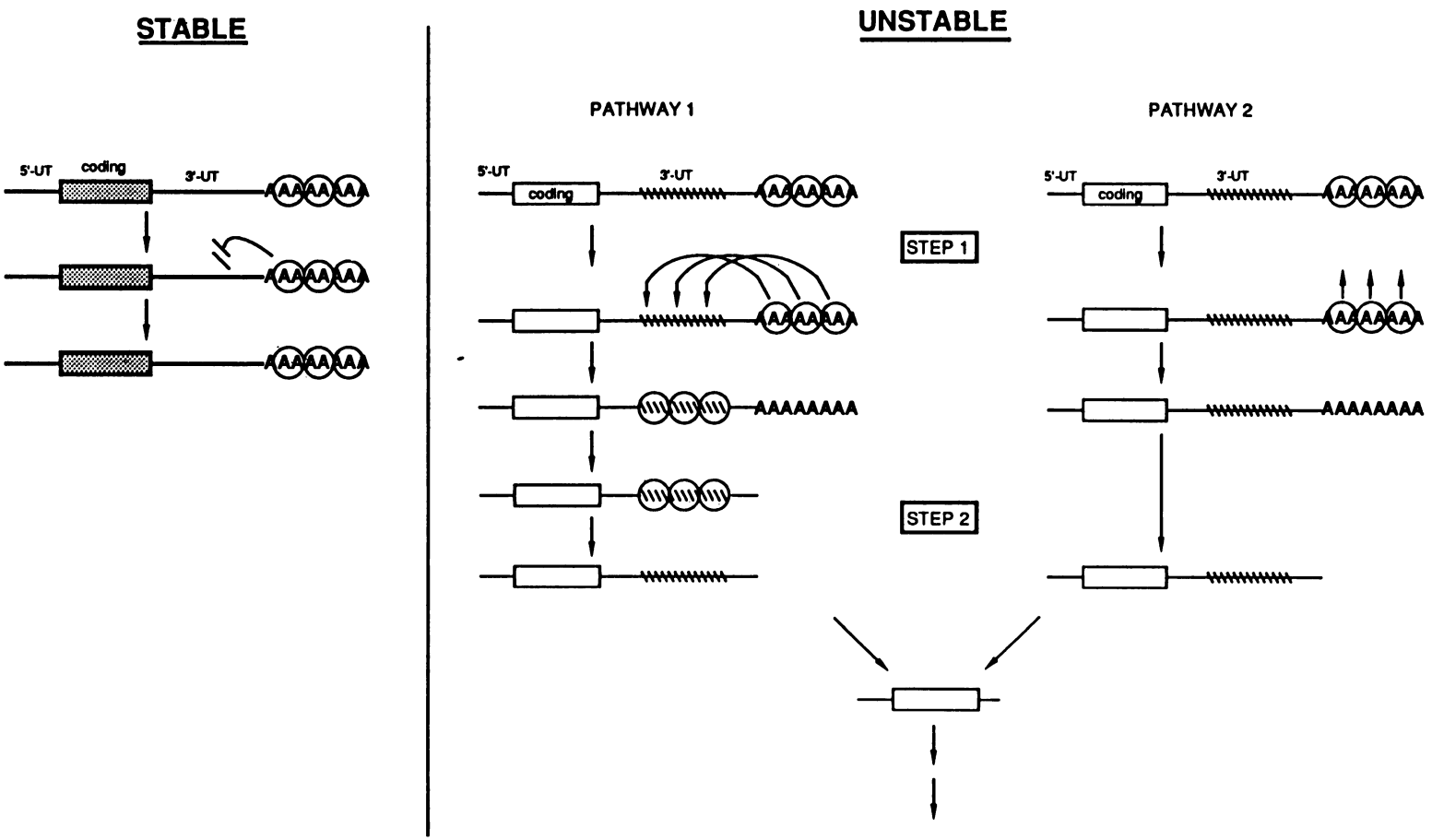

FIG. 12. Model for the role of *cis*-acting mRNA sequences in determining the stabilities of the poly(A)-PABP complex and the mRNA body. The open circles designate PABP. The hatched lines in the unstable mRNA designate sequences that induce PABP to dissociate from the poly(A) tract, thereby increasing the susceptibility of poly(A) to nucleases. Since the stable mRNA is deficient in dissociation-inducing sequences, the PABP stays bound to its poly(A) tract for a longer time, and the mRNA is therefore more stable. Although the diagram shows the dissociation sequences in the 3'-untranslated region, they could be anywhere in the mRNA. The only restriction on these sequences is that they function in *cis*, not in *trans*. They could function in an indirect fashion, for example, by acting as binding sites for cellular factors that affect the stability of the poly(A)-PABP complex. Two pathways for PABP dissociation are shown. In pathway 1 the PABP migrates from the poly(A) to within the mRNA itself. In pathway 2 it simply dissociates from the poly(A). The ability of PABP to dissociate or to migrate probably results from the presence of four separate RNA-binding sites in a single PABP molecule (2, 23, 58, 59). See Discussion for details.

poly(A) tract before poly(A) shortening (and mRNA degradation) can begin.

One testable model is based on the mobility of PABP, that is, its ability to move from one binding site to another (59, 60; see also references 1, 36, and 65). There are two essential aspects of the model (Fig. 12). (i) PABP remains bound for a longer time to the poly(A) tract of a stable mRNA than to that of an unstable one. (ii) The lifetime of the poly(A)-PABP complex is determined by *cis*-acting sequences in the mRNA body. Unstable mRNAs contain sequences (hatched lines in Fig. 12) that somehow promote the dissociation of PABP from the poly(A) tract, while stable mRNAs lack or are deficient in such sequences. As a result of PABP dissociation (step 1), the unprotected poly(A) becomes nuclease susceptible. If it is attacked by nucleases before another PABP molecule can bind to it, the poly(A) shortens and eventually disappears (step 2), leaving a deadenylated mRNA that is more susceptible than its polyadenylated counterpart to further nuclease attack.

This model is consistent with the in vitro data presented here. For example, when PABP dissociation is induced by the presence of excess competitor poly(A), a normally stable mRNA is destabilized (Fig. 1). The model also provides an explanation for how *cis*-acting sequences might affect poly(A)-shortening rates. It thereby links poly(A) metabolism and mRNA turnover with mRNA primary structure, which is the ultimate determinant of mRNA stability. Recent experiments have identified specific sequences which can affect mRNA stability (12, 57, 63, 68), and it would be interesting to know if they do so by affecting the poly(A)-PABP complex. The model shows these sequences in the 3′-untranslated region of the mRNA, but they could just as well be elsewhere. Moreover, the model assumes that there is a limited pool of free PABP in cells. If cells contained a vast excess of PABP, the protein-free poly(A) might bind another PABP molecule so rapidly that poly(A) shortening would rarely occur.

The model shows two possible pathways for PABP dissociation. In the first, PABP migrates away from the poly(A) and reassociates transiently with specific regions in the unstable mRNA. In the second, it dissociates but does not interact further with the mRNA. If reassociation were to occur, it seems unlikely that PABP would bind directly to the mRNA. Although PABP can bind to RNA sequences other than poly(A) (36, 59, 65), a direct (protein-RNA) binding mechanism would require that PABP migrate from a high-affinity to a lower-affinity RNA-binding site. Therefore, it seems more likely that any internal PABP-binding sequences would function indirectly, perhaps as ribonucleoprotein structures in which the protein components act as the inducers of PABP dissociation.

Since some mRNA decay pathways are the same in vitro and in whole cells (see above), we suggest that the poly(A)-PABP complex plays an important role in differential mRNA turnover in cells. Some experiments support this suggestion. For example, poly(A) shortening seems to precede degradation of the body of many cellular mRNAs (13, 20, 25, 39, 40, 52, 68, 70, 73). This observation, coupled with the results presented here, indicates that some mRNAs remain intact until their poly(A) tracts are removed. Recent in vitro experiments with c-*myc* mRNA support this conclusion (11), as do experiments showing that the stability of growth hormone mRNA in cells is increased when its poly(A) tract is elongated (45). Some oocyte microinjection experiments revealed no correlation between stability and poly(A) length for $\alpha_{2\mu}$-globulin mRNA (16), and the apparent rate or extent of poly(A) shortening for actin and tubulin mRNAs in differentiating erythroleukemia cells did not correlate with the rates at which these mRNAs were degraded (34, 42). These results are not incompatible with a poly(A) protection mechanism, because the poly(A)-shortening rate need not be uniform throughout the lifetime of a poly(A) tract. Furthermore, our results and others as well (39, 44, 48) indicate that an mRNA molecule can be protected (stabilized) as long as its poly(A) tract maintains the minimum size (25 to 32 bases) necessary to bind a single PABP molecule.

It seems likely that poly(A) plays more than one role in mRNA metabolism, affecting not only stability but also translational efficiency (46, 47, 62; reviewed in reference 30). Moreover, some mRNAs seem to be just as stable without as with their poly(A) tracts (41, 62, 64). Thus, there are probably multiple determinants of mRNA stability and several degradation pathways as well, depending on the mRNA. The poly(A)-shortening rate might be important for some, but perhaps not all, mRNAs. The stabilities of some mRNAs might be determined by the efficiency with which they are translated, by the structure or intracellular location of the polysome with which they are associated, or by the amount of free end product they produce (14, 15, 24, 43, 49, 67, 71).

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