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Nuclease Activity Associated with Mammalian mRNA in Its Native State: Possible Basis for Selectivity in mRNA Decay

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Polysome and messenger ribonucleoprotein (mRNP) preparations from various mammalian cells contain tightly bound nuclease activity that causes degradation of the mRNA in the preparations. This activity was found to cosediment with all polysome size classes as well as with free mRNPs and to remain associated with the mRNPs released from polysomes by treatment with EDTA. No association with ribosomal subunits was evident. The rates of mRNA degradation were not affected by serial dilution, an indication that enzyme and substrate are tightly associated. β-Globin mRNA in purified reticulocyte polysomes was cleaved at AU sequences in the 3′-terminal region. Cleavages at the same sites occurred when deproteinized reticulocyte RNA was incubated with mouse sarcoma 180 (S-180) polysomes. The S-180 preparations caused additional cleavages, primarily at UG sequences. A P40 mRNA in S-180 polysomes was cleaved primarily in the 3′ noncoding region, but the cleavages in a P21 mRNA were seen in the 5′ noncoding region only. Actin mRNA was cleaved in an internal region, yielding large relatively stable 3′- and 5′-terminal fragments. These data suggest the occurrence of highly specific interactions between one or more mRNA-bound nucleases and individual mRNA species.

The process of mRNA decay represents a major site of control of gene expression. The high degree of stability of species such as globin mRNAs permits accumulation to high levels in the cells and contributes to the high degree of expression of the corresponding genes (2, 20, 27). Highly unstable species, such as oncogene and lymphokines mRNAs (25), can accumulate in a transient fashion because their high decay rates lead to rapid disappearance after cessation of gene transcription. mRNAs with a range of intermediate decay rates are also known. The decay of many mRNAs is subject to regulation by signals that affect the control of expression of the corresponding genes. Induction by steroid hormones and by prolactin involves not only gene activation but also mRNA stabilization (5, 12). Histone mRNAs are relatively stable during the period of DNA synthesis and are destabilized upon completion of this process (13). Tubulin depolymerization provides a signal for rapid tubulin mRNA decay (28), and the transferrin receptor mRNA is destabilized in the presence of excess iron (6, 22).

The wide diversity of individual mRNA decay rates indicates the occurrence of highly specific interactions between critical sites on the mRNA and components of the decay machinery. Structural determinants of mRNA stability have been identified on some mammalian mRNAs, such as the A+U-rich destabilizing sequence on lymphokine and oncogene mRNAs (25), the 3′-terminal stem-loop on histone mRNA that links its decay to DNA replication (19), and the iron-responsive destabilizing element on transferrin receptor mRNA (6, 22). There is very limited information on the nature of cellular components that interact with these structures (23) and none on the manner in which such components might affect decay rates. Moreover, target sites for the initiation of decay remain to be identified, except in the case of the 3′-terminal stem-loop structure on histone mRNA (24).

Knowledge of the nucleases involved in mRNA decay is essential for an understanding of how the decay of individual species can be controlled. Control of degradation by exonucleases could conceivably be achieved by structural features of the mRNA that limit accessibility to 3′ and 5′ termini. Control of endonucleolytic action would require the involvement of highly specific nucleases that recognize unique sites on mRNA molecules. Such enzymes are not known in mammalian cells. The 3′ stem-loop structure of histone mRNA has been shown to be the target of an enzyme, believed to be a 3′ exonuclease, that is loosely bound to ribosomes. It has been suggested that initial cleavages on some mRNAs may be triggered by a ribosome-bound nuclease, which would become active when the ribosome reaches a certain site in the coding region. The suggestion is based on the observation that premature termination of translation of tubulin and histone mRNAs prevents their rapid decay (11, 28). The requirement for translation could also be explained by unmasking of a target site due to unfolding of the mRNA during ribosome movement.

The findings in this report suggest another mechanism for individual mRNA decay control, through activation of a nuclease associated with the mRNA molecules. We have observed that purified polysome preparations contain a nuclease activity that causes degradation of the endogenous mRNA. The activity is subject to inhibition by a soluble cytoplasmic factor. Our evidence indicates that the nuclease activity is associated with mRNA in its nucleoprotein state, not with ribosomal particles. Examination of sites of cleavage in individual mRNA species suggests the occurrence of highly specific interactions between bound enzyme and mRNA molecules. It also appears that enzymes with dif-

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ferent cleavage specificities occur on different mRNA species.

MATERIALS AND METHODS

Preparation of cell extracts and of polysomes. Mouse sarcoma 180 (S-180) ascites cells, maintained by weekly transfer into the peritoneal cavity of CD-1 albino mice, were harvested and incubated in culture medium for 1 h (29). Cells were collected from cultures chilled rapidly to avoid ribosome runoff. They were washed with saline, suspended in three volumes of disruption buffer (100 mM potassium acetate, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 1 mM $MgCl_2$, 2 mM dithiothreitol), and disrupted by passage through a nitrogen pressure cell (Kontes, Vineland, N.J.). The pressure used in this procedure, 600 lb/in^2, was the minimum pressure required for complete cell disruption. This treatment causes breakage of the cells without obvious damage to the nuclei. After centrifugation at 10,000 × *g* for 10 min to remove nuclei and cell debris, the extracts were either used directly for the experiments or stored at −70°C after quick freezing. When extracts enriched in free messenger ribonucleoproteins (mRNPs) were required, the S-180 cells were subjected to a starvation treatment that leads to release of the mRNA from polysomes (4). Rabbit reticulocytes were prepared and lysed as described previously (1).

Polysomes were isolated from the cytoplasmic extracts by centrifugation for 1 h at 100,000 × *g* and were purified by a second centrifugation cycle in the presence of 0.5 M potassium acetate. The preparations contained polysomes as well as free ribosomes and mRNP particles.

Assays for mRNA degradation. Mixtures containing polysomes or mRNPs were incubated at 37°C in disruption buffer. Samples were transferred at measured time intervals into chilled tubes and subjected to deproteinization as follows: the samples were diluted with 4 volumes of ice-cold 0.1 M Tris hydrochloride (pH 9.0) containing 0.5% sodium dodecyl sulfate, mixed with 4 volumes of chilled aqueous phenol, and subjected to phenol and phenol-$CHCl_3$ extraction as described previously (10). It was essential to carry out the deproteinization procedure immediately after addition of the Tris-sodium dodecyl sulfate and the phenol in order to avoid losses of functional mRNA. The RNA was recovered by ethanol precipitation.

Overall mRNA levels were measured by their capacity to direct polypeptide synthesis. RNA samples of up to 3 μg were incubated with the nuclease-treated rabbit reticulocyte lysate as described previously (7), using [^{35}S]methionine as the labeled amino acid. Total incorporation into material insoluble in hot trichloroacetic acid, corrected for endogenous incorporation, was taken as a measure of intact mRNA content. In some experiments, only small amounts of RNA, not sufficient for optical density measurements, were available. In this case, tracer rRNA labeled with [^{3}H]uridine to high specific activity was added to the incubation mixtures before deproteinization, and the amount of material used for the translation assays was determined by the radioactivity in the ethanol pellets.

A simplified assay for mRNA degradation was also used. This involved adding samples of incubation mixtures directly to the translation system (direct assay). In this manner, the activity of mRNA was measured without prior deproteinization. Previous studies have shown that the reticulocyte cell-free system can translate the mRNA in mRNPs and polysomes at least as efficiently as can deproteinized mRNA (4).

For the analysis of individual mRNA species, RNA samples were subjected to Northern (RNA) blot analysis. The samples were denatured by incubation with 50% formamide and 6% formaldehyde and subjected to electrophoresis in 1% agarose gels (16). The material transferred to nitrocellulose sheets was hybridized with nick-translated cDNA probes, and the sheets were washed and subjected to autoradiography. The cDNA probes used for globin, actin, P40, and P21 are described below. A genomic mouse c-*myc* DNA clone was used as probe for c-*myc* mRNA.

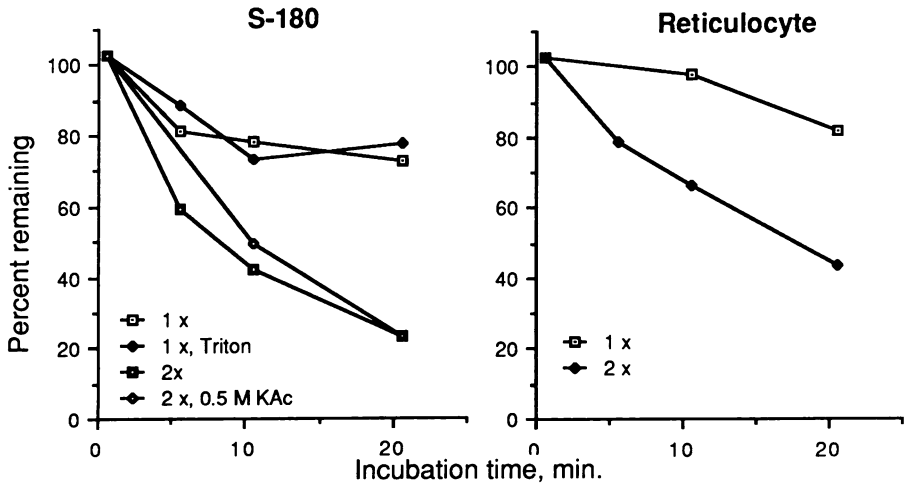

FIG. 1. mRNA degradation in different polyribosome preparations. Mouse S-180 cytoplasmic extract and rabbit reticulocyte lysate were subjected to centrifugation as described in Materials and Methods for the preparation of polyribosomes. Portions of the S-180 extract were centrifuged (1×) in the presence or absence of 0.1% Triton X-100. The preparation obtained in the absence of detergent was recentrifuged (2×) in the presence of either 0.1 or 0.5 M potassium acetate. The reticulocyte preparations were obtained by centrifugation (1×) in the absence of detergent and after recentrifugation (2×) in 0.1 M potassium acetate. Samples of each preparation containing 2 A_{260} units were incubated in 40 μl of disruption buffer. Data are expressed as percent of original mRNA translation activity in RNA isolated after the indicated incubation periods.

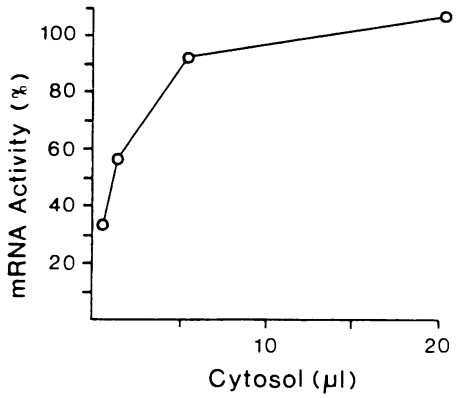

FIG. 2. Effect of the high-speed supernatant fraction of S-180 extract on mRNA degradation in isolated polysomes. Samples of purified (twice-centrifuged) S-180 polysomes (2 A_{260} units, corresponding to the amount present in 50 μl of crude extract) were incubated for 5 min in disruption buffer in the presence of the indicated amounts of the high-speed supernatant (cytosol) fraction. Data represent percent of translation activity remaining after incubation.

Analysis of cleavage sites. Cleavage sites on individual mRNAs were identified by an S1 mapping procedure (8, 15). RNA from incubation mixtures was hybridized with end-labeled restriction fragments of cloned cDNAs, and the fragments protected from S1 nuclease digestion were analyzed by polyacrylamide gel electrophoresis followed by autoradiography. Sites of cleavage were determined from the length of protected probe fragments. Full-length cDNAs for rabbit β-globin (14) and for mouse P40 (21), P21 (9), and β-actin (26) were used for these studies. They contained substantial poly(dT) sequences complementary to the poly(A) tail and could be used for mapping the poly(A) junction and the beginning of the poly(A) sequence on the mRNAs (15).

RESULTS

mRNA degradation in polysome preparations. Polysome preparations obtained from S-180 cytoplasmic extracts by centrifugation at 100,000 × *g* were found to contain a nuclease activity that causes degradation of the mRNA upon incubation at 37°C (Fig. 1). The extent of degradation was measured by comparing the capacity of mRNA isolated from unincubated and incubated mixtures to direct polypeptide synthesis in the reticulocyte cell-free system (see Materials and Methods). The activity was considerably higher in preparations subjected to a second cycle of centrifugation. The nuclease responsible for this activity was firmly associated with particles in the preparations, as indicated by the fact that it was not released by centrifugation in the presence of high salt (Fig. 1). It is apparently not part of some membrane vesicles (such as lysosomes), since the activity was not affected by isolation in the presence of a nonionic detergent (Fig. 1).

The polysome preparations were obtained from extracts that had relatively little activity against endogenous mRNA. This result suggested that the particle-bound nuclease was inhibited by a factor removed during the centrifugation steps. This was verified by examining the effect of the high-speed supernatant fraction (cytosol) on mRNA degradation in polysomes. In the experiment shown in Fig. 2, samples of polysomes equivalent to the amount present in 50 μl of crude cytoplasmic extract were incubated with different amounts of cytosol. Addition of as little as 5 μl of

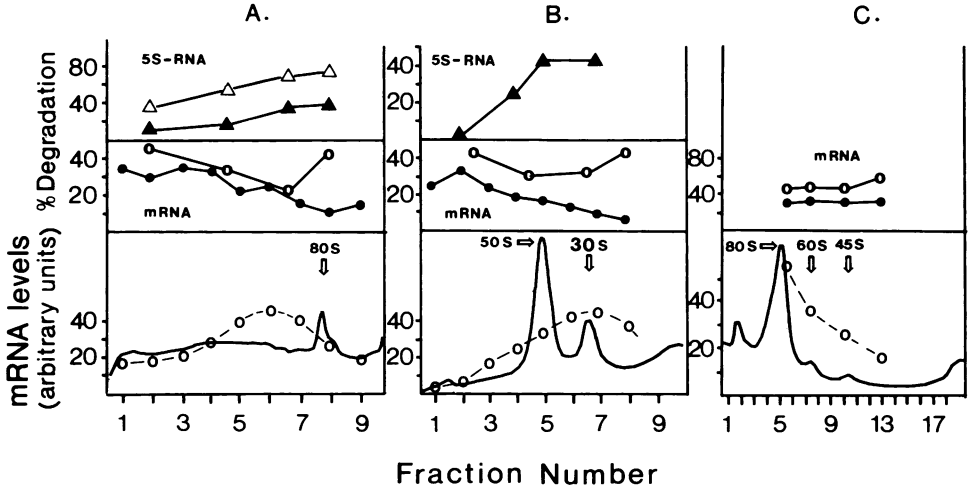

FIG. 3. Sedimentation characteristics of polysomal nuclease activity. In panel A, a purified polysome preparation was subjected to zone centrifugation in a Beckman SW50 rotor for 1 h at 40,000 rpm through a 4.5-ml 10 to 40% linear sucrose gradient with a 0.5-ml 2 M sucrose cushion in 10 mM Tris hydrochloride (pH 7.6)–100 mM KCl–1 mM $MgCl_2$. Samples of 15 A_{260} units of polysomes were used per gradient. Symbols: ——, A_{260} absorption; – – –, distribution of mRNA in gradient, obtained by measuring the capacity of samples of each fraction to stimulate polypeptide synthesis in the reticulocyte translation system (direct assay); ●, nuclease activity against endogenous mRNA in each fraction, obtained by comparing the translation activities of fractions before and after incubation at 37°C for 20 min; ○, same activity in material from pooled fractions centrifuged at 100,000 × *g* for 2 h; ▲ and △, nuclease activity of pooled fractions before and after pelleting, respectively, against ^{32}P-labeled 5S RNA. Assay for 5S RNA degradation consisted of determination of intact RNA left after incubation at 37°C for 20 min, as measured by gel electrophoresis on 15% polyacrylamide–7 M urea, followed by autoradiography and densitometry scanning. In panel B, samples of polysomes in 25 mM EDTA were centrifuged at 49,000 rpm for 110 min through a sucrose gradient without a 2 M sucrose cushion. See above for other details. In panel C, polysome preparations from starved cells (see Materials and Methods) were centrifuged at 49,000 rpm for 90 min through a 10 to 30% sucrose gradient without cushion. See above for other details.

TABLE 1. mRNA degradation in polysome and mRNP fractions[a]

Incubation time (min)	Polysomes				EDTA mRNPs				Native mRNPs					
	Large		80S		50S		30S		60S		45S		30S	
	Incorporation[b]	% Degradation	Incorporation	% Degradation	Incorporation	% Degradation	Incorporation	% Degradation	Incorporation	% Degradation	Incorporation	% Degradation	Incorporation	% Degradation
0	2,210		1,000		1,700		1,270		630		910		1,170	
		86		58		31		38		73		69		60
20	310		420		1,180		780		170		280		470	

[a] Samples of RNA isolated from incubation mixtures were used for translation assays. Large polysomes and 80S represent pooled fractions 1 to 3 and fraction 8 in Fig. 3A, respectively.

[b] Expressed as counts per minute incorporated in acid-soluble material minus endogenous activity.

cytosol (ratio of cytosol to polysomes of 1/10) caused nearly complete inhibition of mRNA degradation. Thus, it appears that the inhibitory factor is present in large excess over the amounts required to neutralize the polysomal nuclease. It is quite likely that the higher nuclease activity of polysome preparations subjected to a second cycle of centrifugation was due to removal of residual soluble inhibitory factor still present in once-centrifuged preparations. In a separate study, the cytosolic inhibitor was partially purified by heparin-Sepharose chromatography and was found to be effective against a variety of endonucleases. It seemed very similar to the placental RNase inhibitor (RNasin; Promega Biotec, Madison, Wis.). The latter was also capable of inhibiting the polysome-bound enzyme.

The rabbit reticulocyte lysate showed the same characteristics as the mouse S-180 extract with respect to degradation of polysomal mRNA. mRNA degradation was very slow in the lysate, but preparations of reticulocyte polysomes isolated by two cycles of centrifugation were quite active in this respect (Fig. 1). The activity was subject to inhibition by the reticulocyte high-speed supernatant fraction (inclusion of 3 μl of this fraction in a reaction mixture containing polysomes from 50 μl of lysate reduced mRNA degradation from 60% to 12%; data not shown). Active polysome preparations were also obtained from mouse erythroleukemia cells (data not shown).

Sedimentation characteristics of nuclease activity in polysome preparations. To identify the particles bearing the polysomal nuclease activity, purified polysome preparations were subjected to zone centrifugation. The distribution of mRNA in centrifugal fractions was measured by determining their capacity to promote polypeptide synthesis in the reticulocyte cell-free system (Fig. 3A). Individual fractions were tested for capacity to inactivate endogenous mRNA. All centrifugal fractions were about equally active in this respect (Fig. 3A). Centrifugation of pooled fractions at 100,000 × *g* brought down the nuclease activity together with the polysomal components (Fig. 3A). The enhanced activity of the light particles after sedimentation (fraction 8) can be explained by removal of residual soluble inhibitor. The initial assay used in these experiments involved adding samples of incubation mixtures directly to the reticulocyte translation

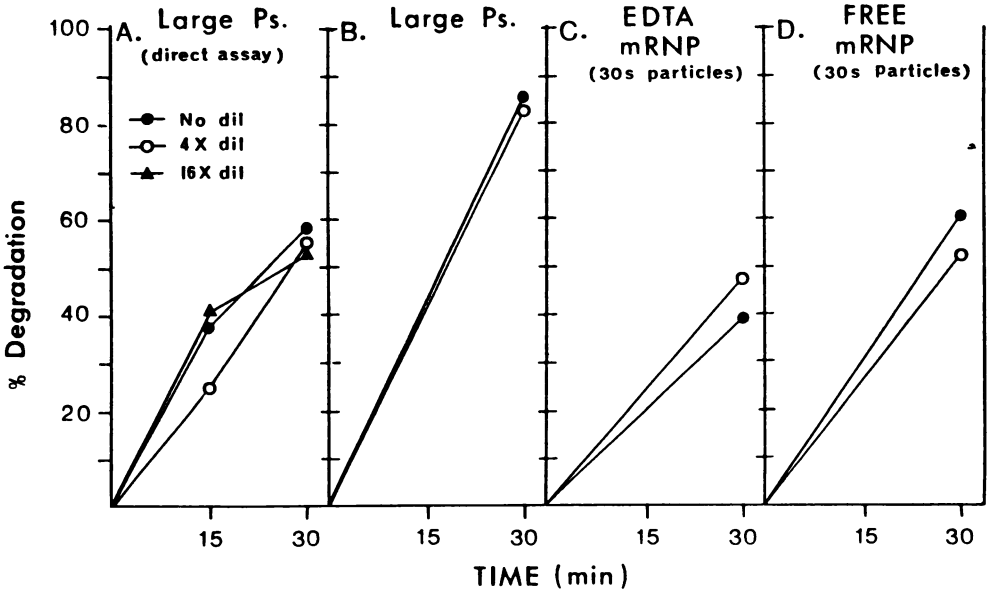

FIG. 4. Effect of dilution on endogenous nuclease activities of polysomal and mRNP fractions. Samples of large polysomes (Ps.; centrifuged pooled fractions 1 to 3 of Fig. 3A), 30S EDTA particles (centrifuged pooled fractions 6 and 7 of Fig. 3B), and free 30S mRNPs (centrifuged pooled fractions 11 to 14 of Fig. 3C) were diluted with disruption buffer to obtain the dilutions indicated and were incubated at 37°C for the indicated time periods. In panel A, samples before and after incubation were assayed for functional mRNA by addition directly to the translation system (direct assay). In panels B, C, and D, the samples were deproteinized before the assay for functional mRNA (see Materials and Methods).

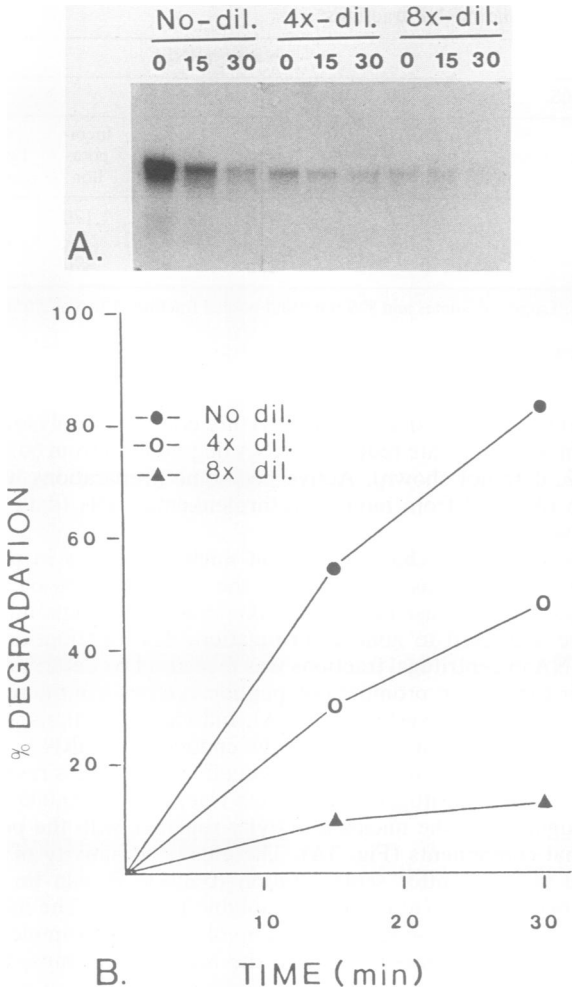

FIG. 5. Effect of dilution on exogenous nuclease activities of polysome fractions. Samples of the repelleted large polysome fraction used for Fig. 4 were mixed with ^{32}P-labeled 5S RNA. Mixtures were diluted with disruption buffer to obtain the dilutions indicated and were incubated for the indicated periods of time. (A) Autoradiograms of incubation mixtures subjected to gel electrophoresis as for Fig. 3; (B) data obtained by densitometry of the autoradiograms.

system (direct assay). The degradation activity of pelleted fractions was also verified by using deproteinized material for the translation assays (Table 1).

The data in Fig. 3A suggested that the nuclease activity might be associated either with the ribosomes or with the mRNA. To distinguish between these two possibilities, polysome preparations were exposed to EDTA before zone centrifugation. This treatment causes dissociation of the ribosomes into 50S and 30S subunits and releases the mRNA as mRNP particles (17). The resulting sedimentation profiles showed that the nuclease activity against endogenous mRNA cosedimented with the mRNA (Fig. 3B). There was no increased activity in the fractions containing the ribosomal subunits, as would have been expected if the nuclease were ribosome bound. The activity was enhanced in the particles after pelleting at 100,000 × g, particularly in the lighter fractions.

The activity of free mRNP particles not generated by EDTA treatment was also examined. Such particles occur normally in cells and can also be released from polysomes in

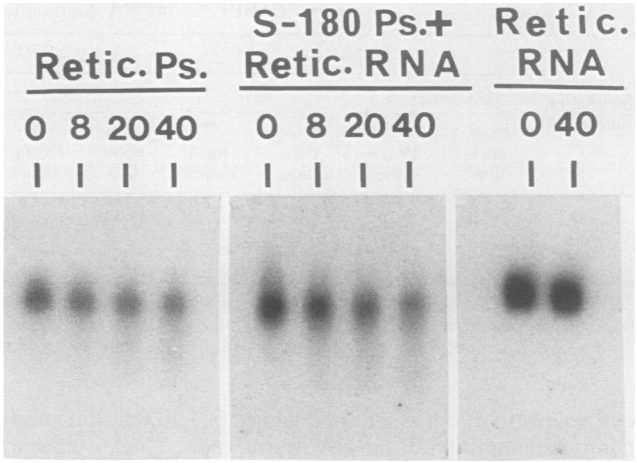

FIG. 6. Degradation of β-globin mRNA in reticulocyte polysomes (Retic. Ps.) and in reticulocyte RNA (Retic. RNA) exposed to mouse S-180 polysomes. Purified reticulocyte polysomes and reticulocyte RNA obtained by deproteinization of reticulocyte lysate (1 A_{260} unit) mixed with purified S-180 polysomes (10 A_{260} units) were incubated for the indicated periods of time (in minutes). RNA isolated from incubation mixtures was subjected to Northern blot analysis, using a rabbit β-globin probe. Reticulocyte RNA was also incubated in disruption buffer without S-180 polysomes.

situ by treatments that cause inhibition of polypeptide chain initiation (18). Preparations from cells subjected to starvation were subjected to centrifugal analysis (Fig. 3C). Although a large proportion of the mRNA still cosedimented with ribosomes, it was possible to analyze small mRNPs (about 30S, present in fractions 12 to 14) well separated from the 45S ribosomal subunit. These mRNPs showed levels of activity against endogenous mRNA similar to those of polysomal and EDTA particles.

Firm association of enzyme with endogenous substrate. Further evidence for the association of the nuclease activity with the mRNA was obtained by comparing the rates of mRNA degradation, using different concentrations of pelleted particles. If the enzyme and the potential target were on the same particle, then the rates of mRNA degradation would be independent of particle concentration. Samples of pelleted particles from polysome gradient fractions (Fig. 3A) were incubated in increasing volumes of buffer so as to obtain dilutions of 1/4 and 1/16. Analysis of mRNA inactivation by the direct translation assay showed that the activity was independent of extent of dilution (Fig. 4). The more precise assay for mRNA degradation, using deproteinized RNA for translation, showed that the activity of polysomes, EDTA mRNPs, and free mRNPs against endogenous mRNA did not change when the incubation mixtures were diluted fourfold (Fig. 4).

Activity of polysomal enzyme on exogenous substrates. The various centrifugal fractions of polysomes and mRNPs were capable of degrading 5S RNA, as measured by loss of intact ^{32}P-labeled RNA by polyacrylamide gel electrophoresis; this activity also remained associated with the particles after centrifugation (Fig. 3). Equivalent fractions from an empty gradient were tested for this nuclease activity. No degradation was observed in this control experiment (data not shown). The activity of fractions from the gradient with EDTA-released particles was proportional to the amounts of mRNA in the fractions and not related to the amounts of ribosomal particles (Fig. 3B). This result provides evidence

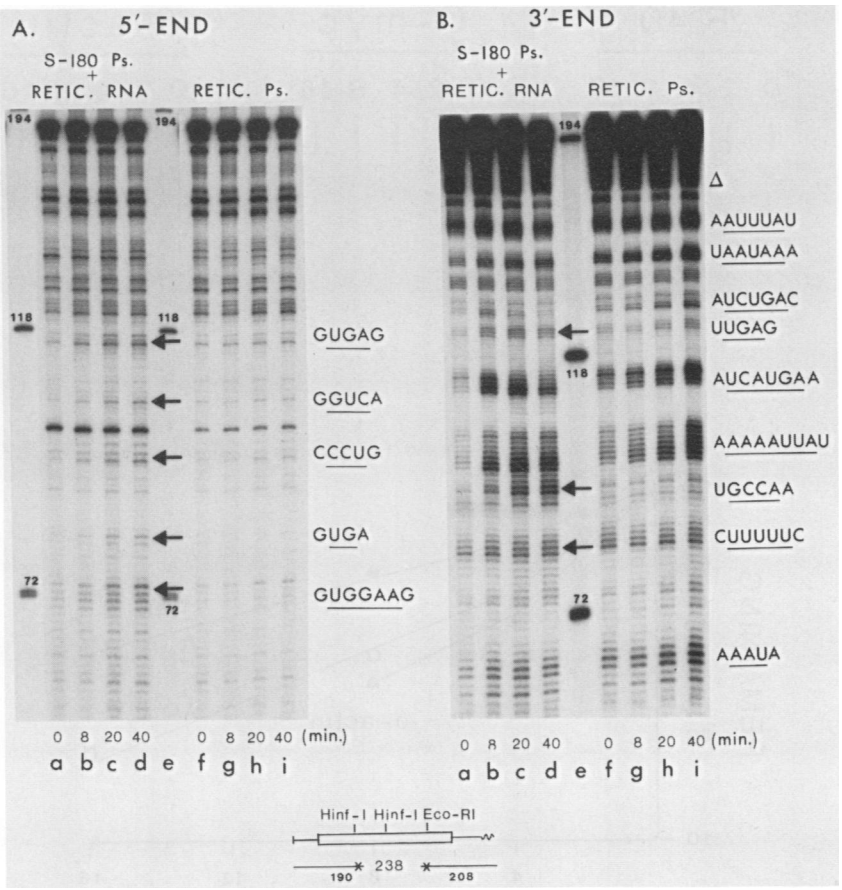

FIG. 7. Cleavage intermediates in β-globin mRNA caused by endogenous and exogenous nuclease activity. RNA samples (2 μg) from the experiment shown in Fig. 6 were subjected to S1 mapping analysis, using end-labeled probes derived from full length β-globin cDNA shown at the bottom. Nucleotide sequences at the right sides indicate sequences around cleavage sites. Symbols: ←, additional cleavages caused by nuclease activity in S-180 polysomes; Δ, poly(A) junction. Numbers below probes denote lengths of probes (in nucleotides) that should be protected by intact mRNA. Lanes e represent size markers. The cDNA diagram shows untranslated regions (——), the coding region (▭), and the poly(A) segment (∿∿∿). Abbreviations are as for Fig. 6.

that the nuclease responsible for 5S RNA degradation was associated with the mRNPs. The effect of dilution on the rates of 5S RNA degradation was also examined, using a polysome fraction. In this case, a strong dependence on concentrations of enzyme and substrate was evident (Fig. 5). This behavior shows that endogenous and exogenous substrates are handled differently by the enzyme or enzymes in the preparations.

Exogenous deproteinized globin mRNA also served as a substrate. Incubation of reticulocyte RNA with purified S-180 polysomes led to considerable degradation of the β-globin mRNA present in the RNA preparation (Fig. 6). There was no degradation when the deproteinized reticulocyte RNA was incubated under the same conditions without S-180 polysomes. Reticulocyte polysomes were capable of degrading their own globin mRNA (Fig. 6).

Nature of cleavages on β-globin mRNA. Incubation of reticulocyte polysomes led to specific cleavages in this mRNA species. The cleavage sites were identified by S1 mapping on RNA isolated from incubation mixtures, using probes for the 3′ and the 5′ ends of the mRNA. Cleavages due to incubation of the polysomes were detected only in the 3′-terminal region (Fig. 7B, lanes f to i). They were all at sites containing an AU sequence. However, one AU site next to the poly(A) junction was not subject to cleavage.

Some fragmentation products due to cleavage at all AU sites were already present in the mRNA of unincubated polysomes (Fig. 7B, lane f). The mRNA in reticulocyte RNA preparations obtained directly from the lysate also showed the occurrence of cleavages at the AU sites (Fig. 7B, lane a). These have been described previously and may represent in vivo cleavages (1). The polysome purification procedure invariably led to some mRNA fragmentation as a result of additional cleavage at some of the sites (compare lanes a and f in Fig. 7B; see also Fig. 8 to 11). This may have been due to loss of the protecting soluble inhibitory factor and nuclease activation during the ultracentrifugations. The 5′ end also showed the occurrence of cleavages in RNA isolated directly from the lysate, but the polysome purification did not cause any additional fragmentation in this region (compare lanes a and f in Fig. 7A). The 5′-terminal region was also not subject to cleavage during incubation of the polysomes (compare lanes f to i in Fig. 7A).

The nuclease activity in S-180 polysomes caused cleavages in exogenous globin mRNA at the same AU sites in the 3′ noncoding region (Fig. 7B, lanes a to d). Three additional cleavages were observed in this region, at sites containing a UG or UC sequence. The exogenous reaction also led to cleavages in the 5′ noncoding region (Fig. 7A, lanes a to d). These were also at sites with a UG or UC sequence. These

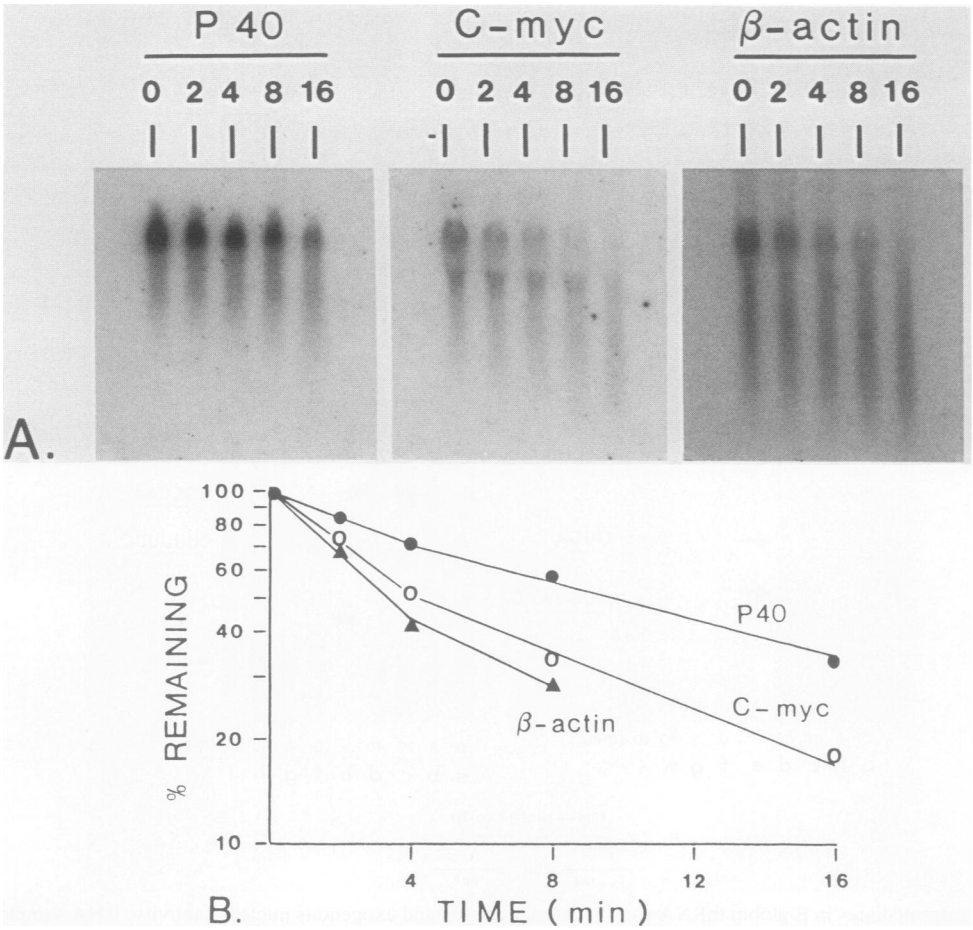

FIG. 8. Degradation of individual mRNAs in incubated S-180 polysomes. (A) Northern blots of RNA from a purified polysome preparation incubated for the indicated periods of time; (B) data obtained by densitometry scanning of the band corresponding to intact mRNA.

results suggest that the S-180 polysomes may carry more than one nuclease with different cleavage specificities and that the reticulocyte polysomes may have only one of these enzymes. They also show that the 5′ noncoding region is accessible only to the exogenous enzyme. It may also be of interest that some sites accessible to RNase T_1 in the 5′- and 3′-terminal regions of β-globin mRNA (1, 14, 15) were not cleaved by the S-180 polysomal nuclease.

A portion of the poly(A) sequence could be mapped with the 3′-end probe because of the occurrence of a corresponding poly(dT) sequence in the cDNA (15). The poly(A) did not seem to be a target for either the endogenous reticulocyte enzyme or the S-180 nuclease activity (see broad band on top of autoradiogram in Fig. 7B).

Cleavage specificity on endogenous S-180 ascites cell mRNAs. The degradation of several endogenous mRNA species was examined in polysomes incubated at 37°C, using Northern blot analysis (Fig. 8). As mentioned above, the mRNAs had already undergone some degradation during polysome purification. The decay in incubated polysomes was nearly exponential. P40 mRNA, a species that is stable in mouse erythroleukemia cells (16), was considerably more stable than actin mRNA during the incubation. However, c-*myc* mRNA, which is far less stable than actin mRNA in intact cells, was somewhat more stable than actin mRNA in the polysome preparation. P21 mRNA, a species that is also stable in mouse erythroleukemia cells, was degraded at about the same rate as P40 mRNA (data not shown).

The cleavages generated in P40 mRNA were analyzed by S1 mapping. Strong cleavages were seen in the 3′-terminal region, primarily at sites containing GC sequences (Fig. 9). Only a few weak cleavages were evident in the 5′-terminal region. Most of these were at AC or ACU sequences. Specific fragmentation due to polysome purification was also evident in this mRNA (compare lanes b and c in Fig. 9).

The P21 mRNA showed a different pattern of cleavages. In this case, the 5′-terminal region was sensitive to the nuclease but the 3′-region was not (Fig. 10). The cleavage specificity was similar to that for the P40 mRNA, with cleavages primarily at GC and AC sequences. The pattern of endogenous cleavages in this mRNA species was quite different from the one generated by exposure of polysomes to RNase T_1, which cleaves at G residues (8).

In the case of actin mRNA, no well-defined cleavage sites were evident (Fig. 11). Although the mRNA molecules were rapidly degraded (Fig. 8), the 5′- and 3′-terminal regions seemed quite stable, as evident from the S1 mapping patterns (Fig. 11A and C). An internal region, mapped by a probe starting at the *Xba*I site, disappeared very rapidly (Fig. 11B). The rate of decay of this region was about the same as that of the intact mRNA (Fig. 8). Northern blot analysis using the 3′-terminal cDNA probe (Fig. 11C) showed the persistence of a major 3′-terminal fragment

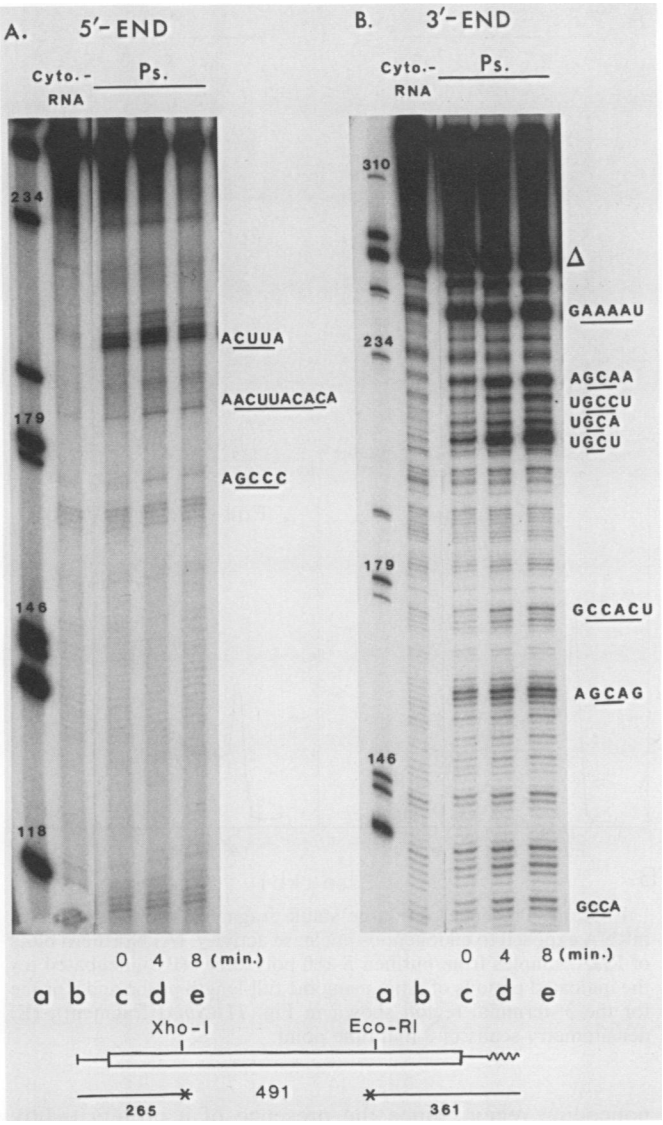

FIG. 9. Cleavage intermediates in P40 mRNA caused by endogenous nuclease activity in S-180 polysomes (Ps.). RNA samples (15 μg) from the experiment shown in Fig. 8 were subjected to S1 mapping analysis, using end-labeled probes derived from P40 cDNA shown at the bottom. Lanes a represent size markers; lanes b show the analysis of RNA isolated directly from S-180 cytoplasmic extract. For other details, see the legend to Fig. 8.

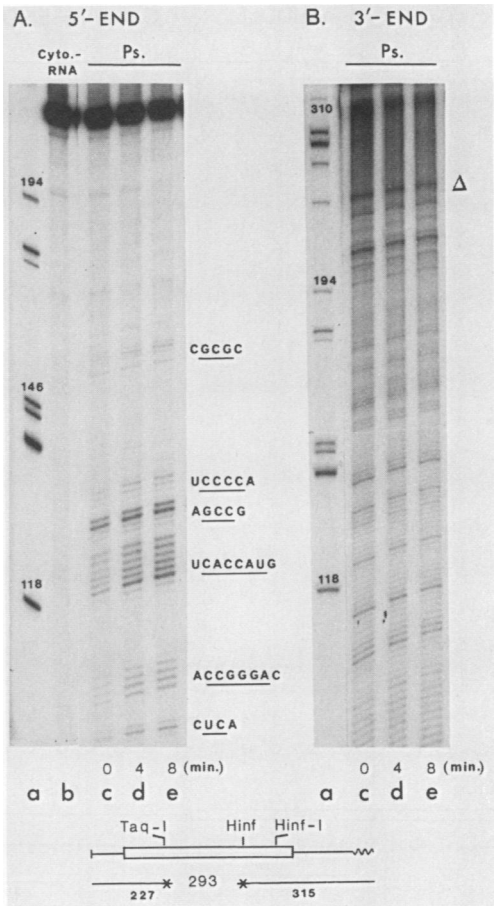

FIG. 10. Cleavage intermediates in P21 mRNA caused by endogenous nuclease activity in S-180 polysomes (Ps.). See legend to Fig. 9 for experimental details.

about 800 nucleotides long (Fig. 12A and B). It appeared that the nuclease activity cleaved an internal region of the actin mRNA, around and downstream of the *Xba*I site. The 3'-terminal portion of the mRNA was also not subject to fragmentation during polysome purification (compare lanes b and c in Fig. 11C).

DISCUSSION

This report provides evidence for the occurrence of a nuclease bound to mRNA molecules in mammalian cells. The nuclease activity degrades the mRNA in purified polysome preparations. Zone centrifugation of polysomes shows that all mRNA-containing fractions are about equally sensitive to degradation. The evidence in Fig. 3 shows that the activity is not associated with ribosomal particles. The activity cosediments with mRNPs released from polysomes by EDTA treatment, and mRNP fractions rich in ribosomal subunits show about the same activity against mRNA as do those with little ribosomal content. It is conceivable that nuclease molecules normally present on ribosomes were transferred to the mRNPs as a result of the EDTA treatment. However, small naturally occurring mRNPs well separated from the 45S ribosomal subunit showed about the same susceptibility to degradation as did the EDTA particles.

Tight association between enzyme and target is indicated by the fact that the rate of mRNA degradation is not affected by dilution of the incubation mixtures. It is possible that most if not all of the mRNA molecules are associated with enzyme, since greater than 50% of the molecules were inactivated in a concentration-independent fashion. The nuclease activity can be attributed to protein associated with the mRNA, since a deproteinized preparation did not undergo degradation when incubated under identical conditions (Fig. 6).

The nuclease activity associated with the mRNA in polysomes handles individual species in a highly specific fashion. β-globin mRNA in rabbit reticulocyte polysomes was cleaved at AU-containing sites in the 3'-terminal region. Most but not all sites carrying this sequence were affected. No cleavages were seen in the 5' noncoding region, even though this region carries some AU sites and is accessible to exogenous nucleases (1, 14). A P40 mRNA in mouse sar-

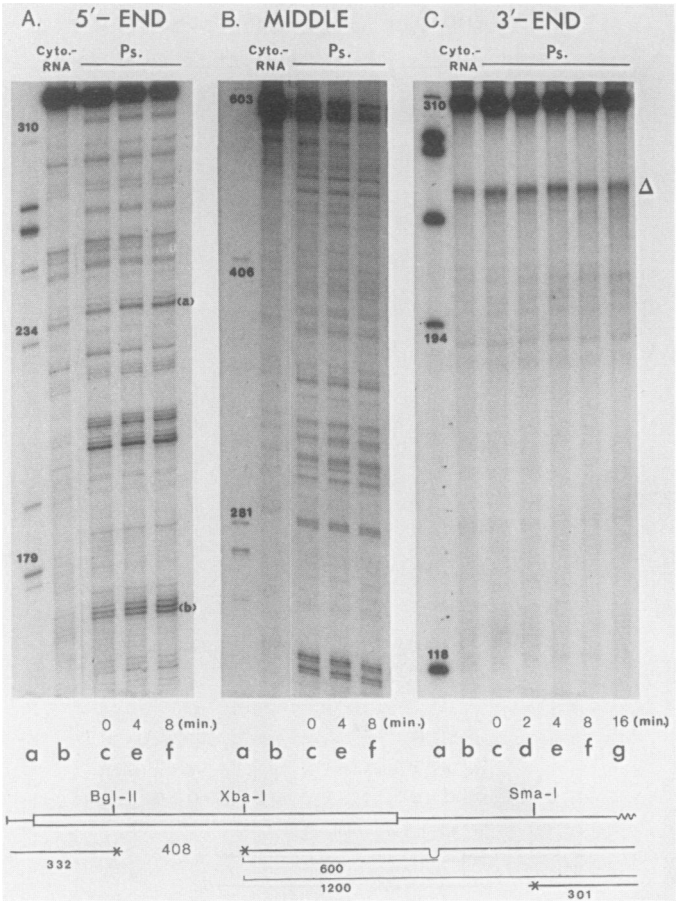

FIG. 11. Cleavages in actin mRNA caused by endogenous nuclease activity in S-180 polysomes (Ps.). Experimental details are as in the legend to Fig. 9. Two minor cleavage sites in the 5'-terminal region, indicated by (a) and (b), were around the sequences UGCG and UAUUC, respectively. Probes used for S1 mapping analysis are shown at the bottom. The probe for the middle region (panel B) consisted of a 3'-end-labeled *Xba*I fragment. This fragment covers a continuous stretch of only about 600 nucleotides because of an apparent sequence divergence in the mouse S-180 actin mRNA and the 3T3 actin cDNA used as probe, in the region indicated by a loop.

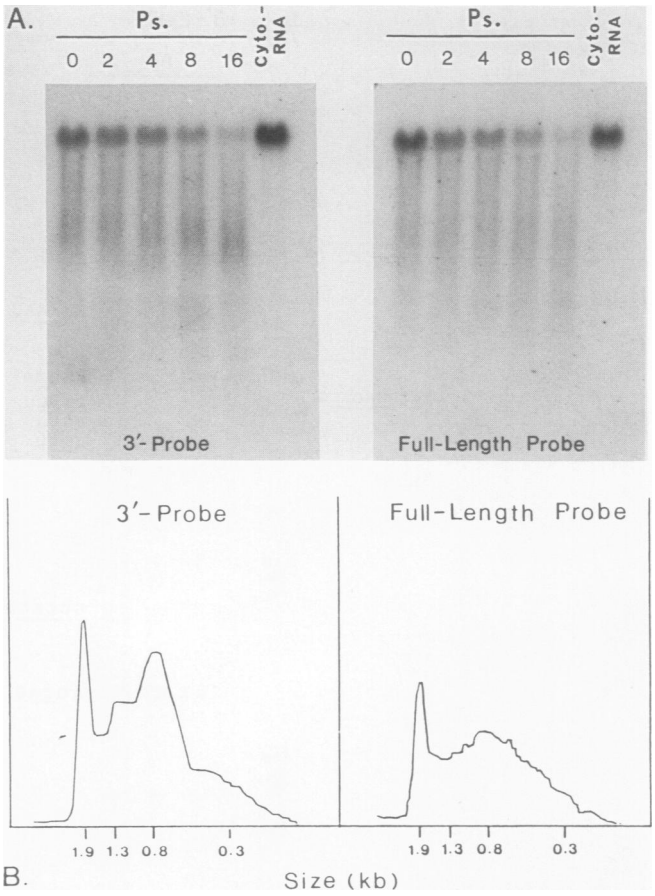

FIG. 12. Presence of a large stable fragment in digests of actin mRNA exposed to endogenous nuclease activity. (A) Northern blots of RNA samples from purified S-180 polysomes (Ps.) incubated for the indicated periods of time, using the full-length probe and a probe for the 3'-terminal region shown in Fig. 11 (*Sma*I fragment); (B) densitometry scans of 8-min time point.

coma ascites polysomes was cleaved primarily at UG and UC sequences in the 3'-terminal region. Another mRNA species, P21, was cleaved in the 5'-terminal region only. Actin mRNA in the same polysome preparation was cleaved internally, releasing 5'- and 3'-terminal segments that remained essentially untouched.

The nuclease activity in S-180 polysomes was also capable of cleaving exogenous deproteinized globin mRNA, causing the same cleavages at AU sites in the 3'-terminal region as those caused by the endogenous reticulocyte enzyme. The S-180 activity, however, caused additional cleavages in both the 5' and 3' regions of the exogenous globin mRNA, primarily at UG sites. This finding suggests that the S-180 mRNA population may carry several nucleases with different cleavage specificities.

The nature of the association between nuclease and mRNA remains to be elucidated. The unique sites of action on individual species could be due to diversity of sites of binding. It could also be that the mRNA species have a common nuclease-binding site and that the cleavage selectivity is caused by unique individual mRNA conformations. It is likely that the nuclease-binding sites are located in the noncoding region, since the presence of a protein tightly bound to the mRNA within the coding region could be expected to interfere with translation.

It is not known whether the association between nuclease and mRNA seen in cytoplasmic extracts also occurs in intact cells. The apparent high degree of specificity of the interaction with individual mRNA species would seem to preclude a random association generated during cell disruption. Also, such artifactual RNA-protein associations tend to be disrupted in high salt. The notion of a natural association between nuclease and mRNA would be strengthened considerably if it were demonstrated that some of the specific cleavages shown here also occur in intact cells. It may be significant that the AU cleavages generated by the reticulocyte polysomal nuclease are also seen in globin mRNA extracted directly from reticulocyte lysates. It has been suggested that the latter cleavages might have been generated in the intact cells (1). Cleavages at AU sites possibly generated in vivo have also been reported for the very-low-density apolipoprotein mRNA (3). More conclusive evidence for the occurrence of such cleavages in intact cells would provide support for the involvement of mRNA-bound nuclease activity in the mRNA decay process.

The occurrence of nuclease molecules tightly associated with mRNA could account for the selectivity of the mRNA

decay process. Such an association could also permit independent regulation of the decay of individual mRNA species. The mRNA-bound nuclease activity is controlled by a soluble inhibitory factor present in large excess in the cytoplasm. Thus, activation of the nuclease would require interference with the enzyme-inhibitor interaction. It is conceivable that the conformation of the mRNA around the nuclease-binding site could affect access to the inhibitor and that shifts in conformation could lead to stabilization or destabilization. Alterations in mRNA conformation due to ribosome movement during translation could affect the inhibitor-nuclease interaction, thus accounting for the linkage between translation and the decay of many mRNA species.

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