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Poly(A) Shortening and Degradation of the 3′ A+U-Rich Sequences of Human c-*myc* mRNA in a Cell-Free System

GARY BREWER* AND JEFFREY ROSS

McArdle Laboratory for Cancer Research and Department of Pathology, University of Wisconsin, 450 North Randall Avenue, Madison, Wisconsin 53706

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The early steps in the degradation of human c-*myc* mRNA were investigated, using a previously described cell-free mRNA decay system. The first detectable step was poly(A) shortening, which generated a pool of oligoadenylated mRNA molecules. In contrast, the poly(A) of a stable mRNA, γ globin, was not excised, even after prolonged incubation. The second step, degradation of oligoadenylated c-*myc* mRNA, generated decay products whose 3′ termini were located within the A+U-rich portion of the 3′ untranslated region. These products disappeared soon after they were formed, consistent with rapid degradation of the 3′ region. In contrast, the 5′ region, corresponding approximately to c-*myc* exon 1, was stable in vitro. The data indicate a sequential degradation pathway in which 3′ region cleavages occur only after most or all of the poly(A) is removed. To account for rapid deadenylation, we suggest that the c-*myc* poly(A)-poly(A)-binding protein complex is readily dissociated, generating a protein-depleted poly(A) tract that is no longer resistant to nucleases.

The rate at which an mRNA is degraded can play a major role in determining its steady-state level. The control of mRNA turnover seems to be especially important for some cell-cycle-regulated genes, such as the histone genes (4, 20, 37, 40, 54, 92), for constitutively expressed (housekeeping) genes (15, 19), and for proto-oncogenes, such as c-*myc* (12, 25, 28, 45, 53).

The c-*myc* gene products are nuclear phosphoproteins of unknown function (3, 34, 38, 39, 76). They are significant not only because they are related to a viral oncogene product (7, 22, 33, 99, 100) but also because they play a role in regulating cell growth and differentiation. c-*myc* mRNA is scarce in quiescent, nontransformed cells but is relatively abundant in cells exposed to mitogens or serum (12, 16, 36, 44, 74, 87, 107). It is usually more abundant in undifferentiated than in differentiated cells (24, 35, 50, 87, 96, 102, 106), but its levels are sometimes transiently elevated during differentiation (63). Finally, there is a correlation between the synthesis of excess or mutated c-*myc* mRNA and neoplastic transformation (2, 51, 52, 69).

It now seems clear that c-*myc* gene expression is regulated, in part, by change in the rate at which its mRNA is degraded. Most of the observed variations in c-*myc* mRNA levels cannot be explained simply on the basis of changes in c-*myc* gene transcription rates (12, 26, 28, 30, 45, 46, 53). Posttranscriptional controls must also function. In fact, direct half-life measurements indicate that c-*myc* mRNA is transiently stabilized in mitogen-treated cells (45, 53).

Our goals are to investigate how c-*myc* mRNA degradation affects c-*myc* gene expression, to delineate the pathway of c-*myc* mRNA degradation, and, ultimately, to determine how its turnover is regulated. Toward these ends, we are using a cell-free system to study mRNA degradation (80). Such a system has several advantages over the use of whole cells. (i) mRNA degradation pathways can be monitored independently of transcription because the only cellular components in the reaction mix are from the cytoplasm. The in vitro approach thus avoids transcriptional inhibitors and complicated chase protocols, which are sometimes necessary when working with intact cells. (ii) mRNA degradation steps occur more slowly in vitro than in cells. As a result, mRNA decay products that might be too unstable to detect with intact cells can be observed in vitro (82). (iii) Extracts from quiescent, growing, and transformed cells can be assayed in vitro to search for *trans*-acting factors that affect mRNA degradation.

The in vitro reaction mix includes polysomes from cells making c-*myc* and other mRNAs. Both the mRNAs and the mRNA-degrading enzyme(s) are bound to the polysomes (70, 81). Previous studies indicated that this and related systems (94) are valid models for investigating mRNA degradation pathways. The rank order of mRNA degradation rates is similar in vitro and in whole cells. β and γ globin mRNAs are stable in both, whereas histone and c-*myc* mRNAs are unstable in both (80). Moreover, the pathway of mRNA degradation in vitro mirrors the pathway in intact cells. Thus, human H4 histone mRNA is degraded 3′ to 5′ in cells and in vitro, with the initial degradation step being the removal of five nucleotides from the 3′ terminus (82). Identical histone mRNA degradation products are observed with RNA from cells and from in vitro reactions, indicating that the in vitro system is especially useful for mapping degradation pathways.

Here we describe experiments indicating that the initial steps in c-*myc* mRNA degradation also occur in the 3′ region. The first detectable step involves poly(A) shortening, with the transient accumulation of oligoadenylated or poly(A)-deficient mRNA containing a maximum of 40 3′-terminal adenylate residues. Soon thereafter, the poly(A)-deficient mRNA is further degraded, generating short-lived intermediates whose 3′ termini are located within an A+U-rich portion of the 3′ untranslated region. Although the 3′ two-thirds of the mRNA is degraded rapidly, the 5′ region is stable in vitro.

* Corresponding author.

MATERIALS AND METHODS

In vitro mRNA degradation. The culturing of human erythroleukemia cells (K562) (28) and isolation of polysomes were performed as previously described (80). Briefly, exponentially growing cells were harvested, washed in cold (4°C) F12 (Ham) medium without serum, and lysed by homogenization in low-salt buffer (1 mM potassium acetate, 1.5 mM magnesium acetate, 2 mM dithiothreitol, 10 mM Tris hydrochloride [pH 7.6]). After removal of nuclei by one low-speed centrifugation, polysomes were pelleted through a 30% sucrose cushion in an ultracentrifuge. The polysomes were resuspended in low-salt buffer and frozen. The components of the cell-free mRNA decay reaction mixtures (25 μl) were identical to those described by Ross et al. (81) and included 0.7 A_{260} unit of polysomes. After incubation at 37°C, total RNA was prepared by phenol extraction. In some experiments, reaction mixtures also contained ^{32}P-labeled mRNA substrates synthesized in vitro by using SP6 RNA polymerase (57).

S1 nuclease mapping and RNase protection. S1 nuclease mapping was performed as previously described (18, 80). Diagrams of the probes are shown in the appropriate figures. For RNase protection analysis, a portion of the c-*myc* gene, from the *Cla*I site in exon 3 to the *Eco*RI site in the 3′-flanking region (see Fig. 2A), was subcloned in the antisense orientation downstream from the SP6 promoter in transcription vector pSP65 (Promega Biotec, Madison, Wis.) to make plasmid pSP65(*Cla*RI). Plasmid DNA was cut with *Ssp*I, and [^{32}P]RNA was transcribed by SP6 RNA polymerase (Promega Biotec) in reactions with [α-^{32}P]UTP (see Fig. 3). RNA (10 μg) from cell-free reactions was hybridized to 9 × 10^5 cpm of antisense probe in 80% formamide–0.48 M NaCl–10 mM EDTA–10 mM Tris hydrochloride (pH 7.5) at 25°C for 18 h. The reaction mixtures were then treated with RNases P1 (Calbiochem-Behring, La Jolla, Calif.; 2.5 μg/ml) and T_1 (Sigma Chemical Co., St. Louis, Mo.; 1 μg/ml) in 0.18 M NaCl–6 mM EDTA–3 mM Tris hydrochloride (pH 7.5) for 1 h at 20°C. RNA was phenol extracted, concentrated by ethanol precipitation, and analyzed in 6% polyacrylamide gels containing 8.3 M urea.

RNase H-RNA blotting. A 10-μg portion of RNA was hybridized under the conditions of Mercer and Wake (58) to 0.5 μg of deoxyoligonucleotide 5′-dCAAGTTCATAGGT GATTGCTC-3′, which is complementary to c-*myc* mRNA sequences including nucleotides (nt) +1931 to +1952 (synthesized at the University of Wisconsin Biotechnology Center, Madison; see Fig. 5C). In some reactions RNAs were also hybridized with 0.3 μg of oligo(dT)$_{12-18}$ (P-L Biochemicals, Inc., Milwaukee, Wis.). The hybrids were incubated with 0.8 U of RNase H (P-L Biochemicals) in 28 mM $MgCl_2$–25 mM KCl–0.5 mM EDTA–20 mM Tris hydrochloride (pH 8.0) at 37°C for 30 min. RNA was phenol extracted, ethanol precipitated, and electrophoresed in a 2% (wt/vol) agarose gel containing 2.2 M formaldehyde. The RNA was transferred to a Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, Calif.), air dried for 30 min, and then irradiated with 254-nm light for 1.5 min with a hand-held lamp (Ultra-Violet Products, Inc.; 21). The membrane was prehybridized for 4 h at 65°C in 0.5 M sodium phosphate (pH 7.0)–1% bovine serum albumin (Pentax fraction V)–1 mM EDTA–7% sodium dodecyl sulfate–45 μg of tRNA per ml–100 μg of denatured calf thymus DNA per ml. A ^{32}P-labeled RNA probe (see below) was added (3 × 10^5 cpm/ml), and the blot was hybridized for 14 h at 65°C (21). It was then washed six times at 65°C in 40 mM sodium phosphate (pH 6.8)–1 mM EDTA–1% sodium dodecyl sulfate and autoradiographed with an intensifying screen.

The [^{32}P]RNA probe used for the experiment for which results are shown in Fig. 5 was transcribed from pSP65 (*Cla*RI) cut with *Nsi*I. The *Nsi*I site is located 11 nt downstream from the 21-mer hybridization site, and the probe is complementary to the 3′-terminal 400 nt of c-*myc* mRNA [to the second poly(A) addition site]. The [^{32}P]cDNA probe used for the experiment for which results are shown in Fig. 7 was synthesized by the random primer method of Feinberg and Vogelstein (32) from a human γ globin cDNA clone, JW151 (103).

Preparation of ^{32}P-labeled mRNA substrates. Plasmid pCLAR1 (*Cla*I site in c-*myc* exon 3 to the *Eco*RI site in the 3′-flanking region) was digested with *Nsi*I and *Afl*II (Amersham Corp., Arlington Heights, Ill.), and the ends were made blunt with T4 DNA polymerase. The 400-base-pair (bp) *Nsi*I-*Afl*II fragment corresponds to most of the c-*myc* mRNA 3′ untranslated sequence. [The *Afl*II site at the 3′ end is located at the most distal poly(A) addition site (see Fig. 4, caret 4).] This fragment was purified from an agarose gel and ligated with plasmid pSD3, which had been linearized with *Sma*I. [pSD3 is a pSP65 derivative containing the SP6 promoter followed by an *Eco*RI-*Sac*I-*Sma*I linker and an 85-bp "poly(A)" tract; it was provided by P. Good, University of Wisconsin, Madison.] The resulting plasmid was pMYCSD3. In step 2, plasmid pM1-11 (c-*myc* cDNA plasmid; 60) was digested with *Bam*HI (at the P2 promoter of the c-*myc* cDNA) and *Nsi*I (in exon 3). The ends were made blunt with T4 DNA polymerase, ligated to *Eco*RI linkers, and digested with *Eco*RI. The 1,800-bp *Bam*HI-*Nsi*I fragment was purified from an agarose gel and ligated to *Eco*RI-digested pMYCSD3 to yield plasmid pGB1023. pGB1023 thus contains the SP6 promoter followed by the c-*myc* cDNA from cap site P2 to the most distal 3′ end of poly(A) site 2 (see Fig. 4). In the final construction, *Eco*RI and *Sac*I sites replace the *Nsi*I site in exon 3.

To prepare β globin and c-*myc* ^{32}P-labeled mRNA substrates, pSPKβ/c (a gift from R. Spritz, University of Wisconsin, Madison; 80) and pGB1023, respectively, were linearized with *Hin*dIII. DNA was transcribed by SP6 RNA polymerase in a reaction mixture that included the synthetic cap analog GpppG, 100 μM unlabeled UTP, and [α-^{32}P]UTP (Amersham; 410 Ci/mmol). The reaction mixtures were phenol extracted and ethanol precipitated.

RESULTS

Degradation of the c-*myc* mRNA 3′-terminal region. Northern blotting (RNA blotting) was performed to assess the general nature of the c-*myc* degradation pathway and to determine whether this labile mRNA was degraded in a random or nonrandom fashion. The appearance of discrete decay products would indicate nonrandom degradation. Furthermore, blotting data should indicate whether the initial nucleolytic cleavages occurred in the midportion of the molecule or near one or both termini.

Cell-free mRNA decay reaction mixtures were incubated for various times (see Materials and Methods), after which RNA was purified, electrophoresed, transferred, and hybridized to a full-length c-*myc* cDNA probe (60). A heterogeneous band ranging from approximately 2,200 to 2,400 nt was observed with RNA from unincubated reaction mixtures (Fig. 1, lane 2, bracket). The band was broad because c-*myc* mRNA is a mixture of molecules, with alternate cap sites (P1 and P2) and polyadenylation sites and with poly(A) tracts of

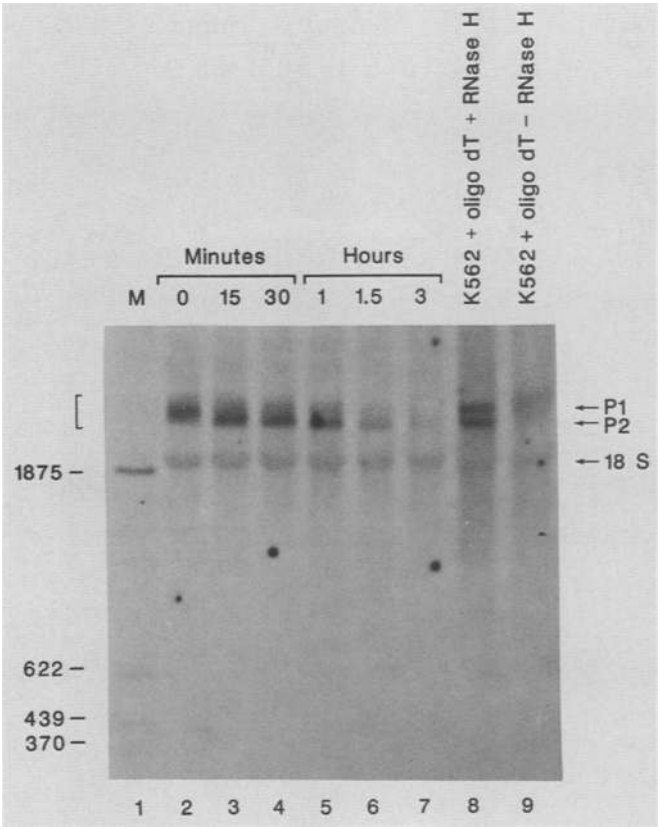

FIG. 1. Analysis of degradation of c-*myc* mRNA in vitro by Northern blotting. Cell-free mRNA decay reaction mixtures containing 0.7 A_{260} unit of polysomes were incubated for the indicated times. Total RNA was extracted, and 10 μg was electrophoresed in a 1% agarose gel, transferred, and hybridized by the method of Church and Gilbert (21) to a ^{32}P-labeled, full-length c-*myc* cDNA plasmid, pM1-11 (kindly provided by G. Ju, Hoffmann-LaRoche Inc.). The blot was exposed for 24 h with an intensifying screen. Lane 1, Markers (pBR322 [^{32}P]DNA cleaved with *Hae*II and kinased; fragment sizes in nucleotides are noted on the left); lanes 2 to 7, RNAs from cell-free reaction mixtures incubated for the indicated times; lane 8, Total RNA (10 μg) from K562 cells annealed with excess oligo(dT), treated with RNase H, electrophoresed, and blotted; lane 9, same as lane 8, but RNase H omitted. 18S, Position of 18S rRNA; P1 and P2, the two major c-*myc* mRNA cap sites. The bracket indicates c-*myc* mRNA.

variable lengths (see below). Poly(A) was a major source of the heterogeneity, because discrete bands were observed when the RNA was treated with oligo(dT) and RNase H before electrophoresis (lane 8, P1 and P2). The intensity of the c-*myc* band decreased during the incubation period (lanes 3 to 7), confirming that the mRNA was degraded rapidly. In contrast, γ globin mRNA was stable, as previously described (80; see Fig. 2 and 7). The average size of the c-*myc* mRNA also diminished gradually during the incubation, indicating that degradation was nonrandom and that initial nucleolytic events had occurred near one or both termini, not in the middle. The 18S band was present in each lane and resulted from cross-hybridization of the probe to 18S rRNA.

Subsequent experiments were designed to detect nucleolytic cleavages in the c-*myc* 3′ region. We focused on this region because it is the primary degradation site of other mRNAs (80, 82, 94). Equivalent amounts of total RNA from cell-free reactions were first analyzed by S1 nuclease mapping, using a 3′ ^{32}P-labeled DNA probe complementary to most of exon 3. RNAs from K562 cells and from unincubated cell-free reaction mixtures protected two major bands, representing c-*myc* polyadenylation sites 1 and 2 (Fig. 2A, lanes 1 and 2, arrows). The intensity of each band decreased at a similar rate during the incubation. Therefore, early steps in c-*myc* mRNA degradation occur near the 3′ terminus. The 3′ region of a stable control mRNA, γ globin, was stable in the reactions (Fig. 2B).

Several new c-*myc* bands appeared after 15 min to 1 h of incubation (Fig. 2A, bracket). They migrated beneath the full-length protected band and were not observed with RNA from unincubated (0 min) reaction mixtures. Their intensities increased from 15 min to 1 h and then decreased during the subsequent 4 h. These bands thus corresponded to short-lived degradation products. On average, they became smaller during the incubation, indicating that degradation had occurred in a 3′-to-5′ direction.

Since the S1 mapping probe protected such a large portion of c-*myc* mRNA, it was not useful for analyzing the structure of the degradation products. Therefore, to confirm the S1 nuclease results and to map the 3′ termini of the degradation products more precisely, an RNase protection assay was performed. RNAs from cell-free mRNA decay reactions were annealed with a uniformly-labeled, 620-nt [^{32}P]RNA probe complementary to the 3′-terminal 200 nt of c-*myc* mRNA (Fig. 3). RNA from unincubated reaction mixtures protected four major bands (Fig. 3, pA_2, and Fig. 4, carets). Identical bands were observed with polyadenylated c-*myc* mRNA purified by oligo(dT)-cellulose chromatography (data not shown). Therefore, each band corresponded to a different poly(A) addition site. The intensities of these bands diminished at approximately equivalent rates during the incubation, indicating rapid degradation of the 3′ region. As the intact mRNA disappeared, smaller bands (Fig. 3, bands I, II, and III) appeared. Bands I to III were not prominent with RNA from unincubated reaction mixtures, were smaller than the intact mRNA bands, and were unstable. Therefore, they represent the transient degradation products. A trace of band I fragments was visible with time zero RNA, which might be expected since some c-*myc* mRNA molecules would be undergoing degradation at the time the K562 cells were lysed. The quantity of each degradation product increased and then decreased at approximately 30-min intervals, in the order I → II → III, confirming a sequential 3′-to-5′ degradation pathway. Their 3′ termini mapped to a particularly A+U-rich portion of the 3′ untranslated region (Fig. 4, arrows). Similar A+U-rich regions, containing some of the signals necessary for rapid degradation of some mRNAs, are characteristic of one class of labile mRNAs and include a consensus sequence, UUAUUUAU (17, 88). Human c-*myc* mRNA contains one match of 7 of 8 nt with this sequence (Fig. 4, underline). The 3′ termini of the degradation products were located 30 to 60 nt 3′ of the consensus site.

Rapid loss of poly(A) from c-*myc* mRNA. The results shown in Fig. 2 and 3 indicate that cleavages in the 3′ region occurred at early reaction times. However, in these and other experiments, there was a 5- to 15-min lag before the degradation products began to accumulate (for example, Fig. 3, lanes 6 to 8). One explanation for this observation is that nuclease attack within the 3′ region is contingent upon some prior degradation step.

To determine whether the lag represented the time required for poly(A) shortening, we devised a technique to monitor degradation of both the poly(A) and the 3′ end (Fig.

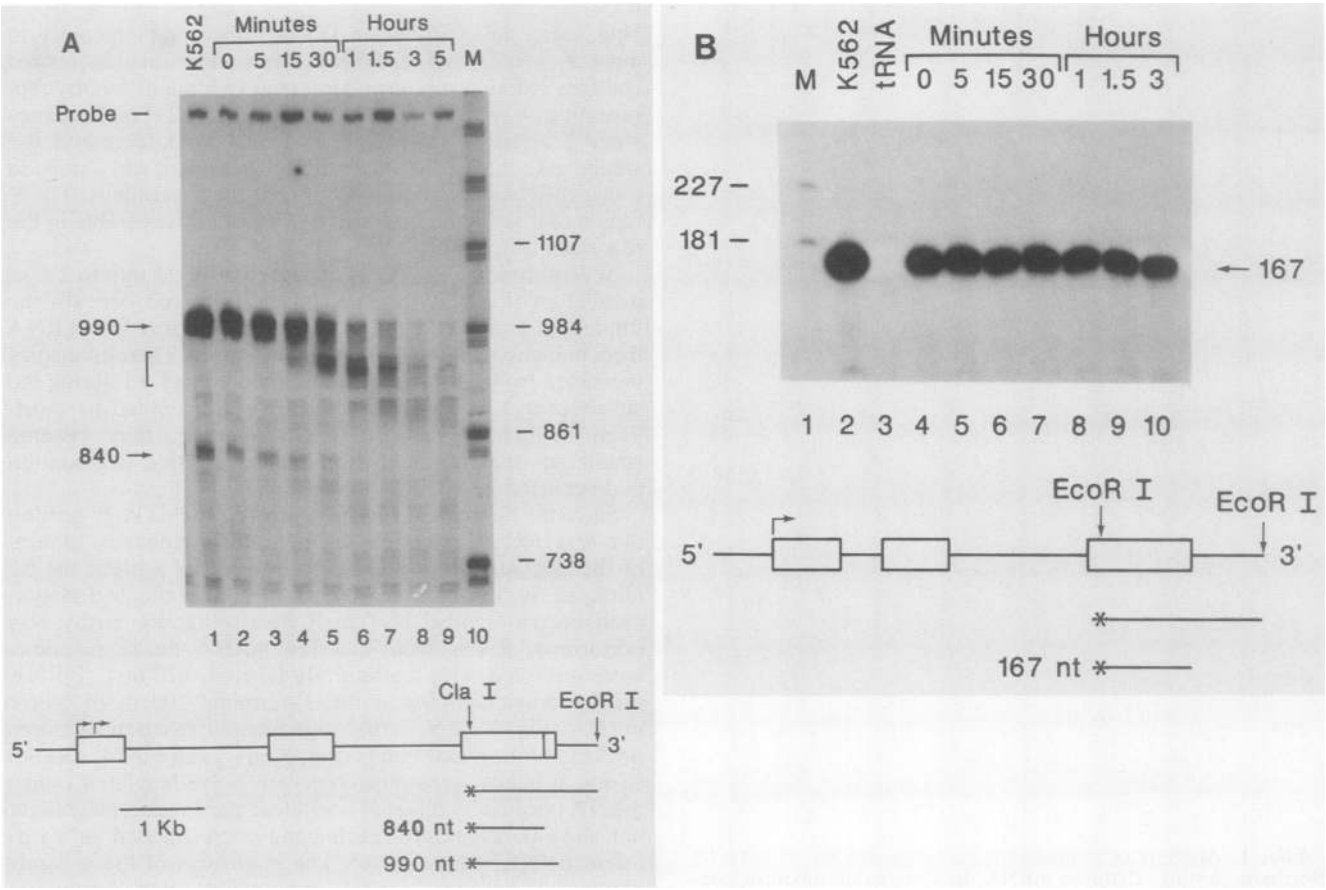

FIG. 2. Analysis of degradation of the 3'-terminal region of c-*myc* mRNA and stability of γ globin mRNA in vitro by S1 nuclease mapping. (A) c-*myc* mRNA. RNA (30 μg) from K562 cells or from cell-free reactions was incubated with a 1,300-bp *Cla*I-*Eco*RI probe that was 3' ^{32}P labeled at the *Cla*I site in exon 3 (see diagram). S1 nuclease treatment and electrophoresis were performed as previously described (18). The gel was exposed for 14 days without an intensifying screen. Lane 1, Total RNA from exponentially growing K562 cells; lanes 2 to 9, RNAs from cell-free reaction mixtures incubated for the indicated times; lane 10, markers (123-bp DNA ladder; the sizes in nucleotides of some of the bands are noted on the right). The bands corresponding to c-*myc* poly(A) site 1 and site 2 mRNAs (840 and 990 nt, respectively) are noted on the left. The bracket indicates c-*myc* mRNA degradation products. A diagram of the human c-*myc* gene is shown at the bottom. ——, 5'- and 3'-flanking regions and intervening sequences; □, exons. The vertical line within exon 3 marks poly(A) site 1. The arrows above exon 1 indicate the two major cap sites, P1 and P2. The lines beneath exon 3 represent the 1,300-bp *Cla*I-*Eco*RI probe and the two fragments protected by undegraded c-*myc* mRNA [poly(A) sites 1 and 2]. The asterisk indicates the labeling site. (B) γ globin mRNA. The same RNAs used in panel A were analyzed with a 3' ^{32}P-labeled *Eco*RI-to-*Eco*RI probe from the 3' region of human γ globin mRNA (80). Each hybridization reaction mixture contained 1 μg of RNA, and the gel was exposed for 3 days with an intensifying screen. The probe migrated in the upper portion of the gel, which is not shown. Lane 1, Markers (kinased fragments of pBR322 DNA cleaved with *Hae*II; sizes in nucleotides are noted on the left); lane 2, 1 μg of total RNA from exponentially growing K562 cells; lane 3, 1 μg of *E. coli* tRNA; lanes 4 to 10, RNAs from cell-free reaction mixtures incubated for the indicated times. The arrow indicates the location of the full-length protected band. A diagram of the human ^{G}γ globin gene is shown at the bottom. The symbols are the same as for panel A.

5C). A 21-nt oligomer complementary to a sequence located 400 nt 5' of poly(A) site 2 was annealed to RNAs from the same cell-free reactions analyzed in the experiment for which results are shown in Fig. 3. The hybrids were treated with RNase H, the RNA fragments were electrophoresed and blotted, and the blot was hybridized with a probe from the c-*myc* 3' region. One advantage of this technique over direct blotting of the RNA was that degradation products were easier to resolve from intact mRNA because the affected region (the 3'-terminal 400 to 600 nt) was cleaved away from the rest of the 2,200-nt mRNA. It also had several advantages over S1 mapping and RNase protection. First, the oligonucleotide was complementary to a G+C-rich region, thus avoiding possible artifactual cleavage of DNA-RNA hybrids rich in A, T, and U residues. Second, the fate of the poly(A) could be monitored.

With this method, c-*myc* mRNA from unincubated reaction mixtures generated heterogeneous fragments ranging from 400 to 600 nt long (Fig. 5A, lane 1, bracket). When the same mRNA was annealed with both the 21-mer and oligo(dT) and then treated with RNase H, a discrete 400-nt band was generated (Fig. 5A, lane 8, long arrow). It corresponded to deadenylated poly(A) site 2 c-*myc* mRNA. [Since poly(A) site 1 mRNA accounts for only 5% of c-*myc* mRNA (Fig. 2A), its deadenylated product was not observed in this autoradiograph.] Taken together, these data indicate that the heterogeneous smear in lane 1 was derived from undegraded c-*myc* mRNAs with different poly(A) tract lengths.

During the incubation the smear became less diffuse and smaller than at 0 min, indicating poly(A) shortening. The relationship between the disappearance of the smear and the appearance of smaller RNAs was assessed by scanning densitometry. Most of the c-*myc* mRNA from unincubated reaction mixtures was larger than deadenylated mRNA,

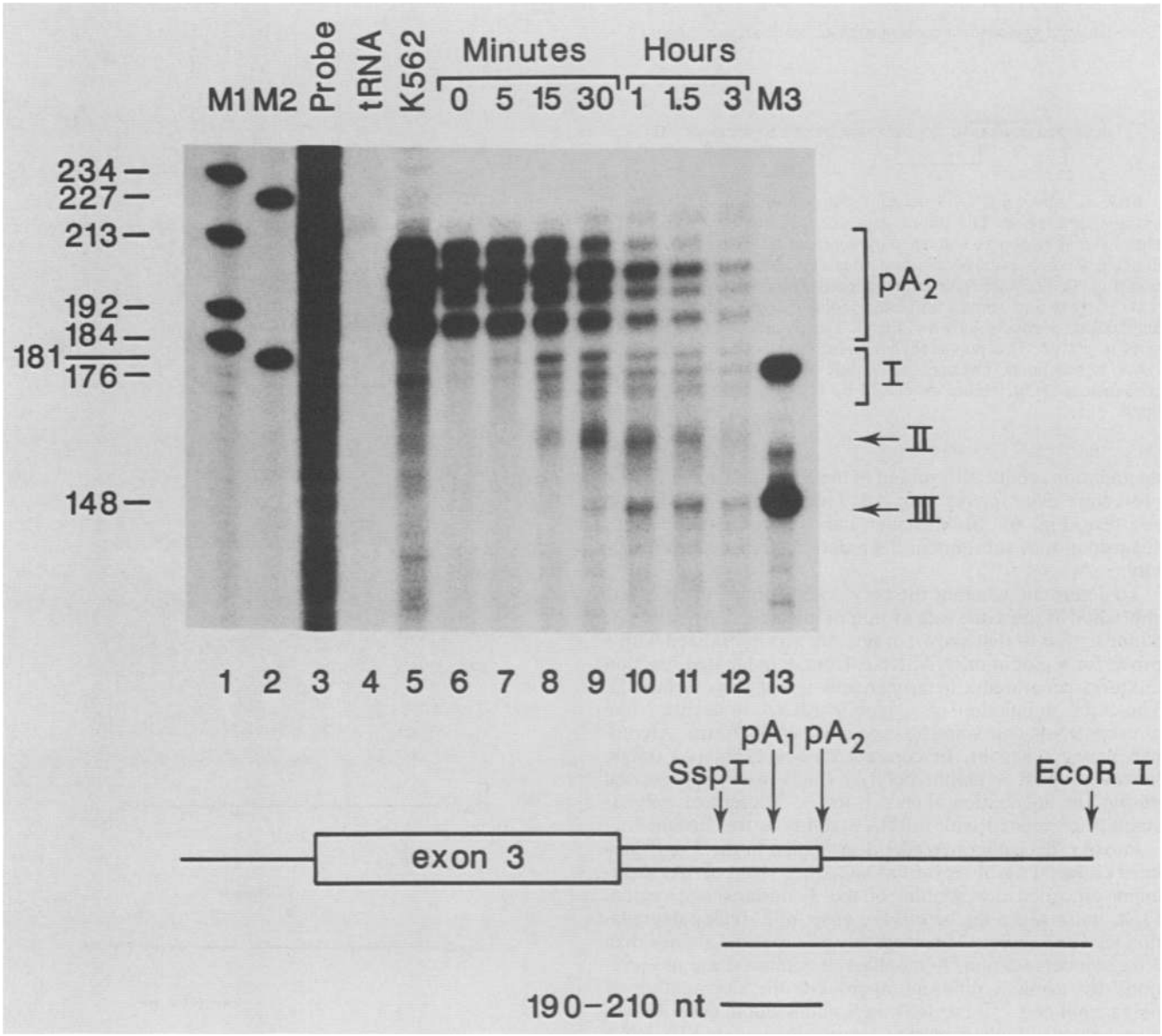

FIG. 3. Analysis of degradation of the 3'-terminal region of c-*myc* mRNA in vitro by RNase protection. RNA (10 μg) from cell-free reactions was annealed with a uniformly labeled, 620-nt [^{32}P]RNA probe extending from the *Ssp*I site in c-*myc* exon 3 to the *Eco*RI site in the 3'-flanking region (see diagram). RNase treatment and electrophoresis of the protected fragments were performed as described in the text. The gel was exposed for 10 days with an intensifying screen. Lanes 1 and 2, Markers (pBR322 [^{32}P]DNA cleaved with *Hae*III and *Hae*II, respectively; sizes in nucleotides are noted on the left); lane 3, RNA probe input, no RNase treatment (most of the probe migrated in the upper portion of the gel, which is not shown); lane 4, 10 μg of *E. coli* tRNA; lane 5, 10 μg of total RNA from exponentially growing K562 cells; lanes 6 to 12, RNAs from cell-free reaction mixtures incubated for the indicated times; lane 13, markers (uniformly labeled [^{32}P]RNAs [176 and 148 nt] synthesized in vitro with SP6 RNA polymerase from pSP65 DNA cleaved with appropriate enzymes). pA_2 indicates the bands corresponding to undegraded poly(A) site 2 mRNA molecules. [Poly(A) site 1 mRNA was not detected with this probe because the fragments it could protect are so small.] I, II, and III, mRNA degradation products (Fig. 4). A diagram of the 3' region of the human c-*myc* gene is shown at the bottom. The large open box is the translated region of exon 3, and the small open box is the 3' untranslated region. The two poly(A) addition sites and the *Ssp*I and *Eco*RI sites used to generate the probe are noted. The long straight line is the uniformly labeled probe; the short straight line is the region of the probe protected by undegraded poly(A) site 2 c-*myc* mRNA.

consistent with poly(A) size heterogeneity (Fig. 6, compare bottom and top tracings). Poly(A) shortening was evident after only 5 min, which was before the time that transient degradation products had begun to accumulate (compare Fig. 5A, lane 2, with Fig. 3, lane 7). Significant shortening occurred between 5 and 15 min. These results thus indicate that poly(A) shortening precedes nuclease attack within the 3' untranslated region. Control experiments revealed that the appearance of specific bands in these gels required both the 21-mer (Fig. 5B, lanes 2 and 3) and RNase H (lanes 8 and 9).

A band that was slightly larger than deadenylated mRNA appeared between 15 and 30 min of incubation (Fig. 5A). It could correspond to mRNA whose poly(A) was shortened but not completely removed, as observed in decay reactions with ^{32}P-labeled c-*myc* mRNA (see below and Fig. 8). Lower-molecular-weight bands that appeared between 30 min and 1.5 h of incubation could correspond to the unstable

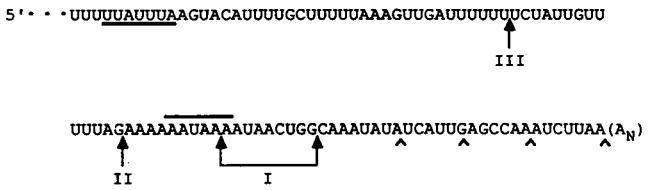

FIG. 4. c-*myc* mRNA 3′ termini and degradation sites in the 3′ untranslated region. The 100-nt sequence from the c-*myc* mRNA 3′ untranslated region is shown (derived from reference 34). Carets indicate the four poly(A) addition sites detected by RNase protection (Fig. 3). They are referred to collectively as poly(A) site 2 in the text. Arrows and roman numerals indicate the 3′ termini of the degradation products shown in Fig. 3. The accuracy of the indicated sites is ±3 nt. The polyadenylation hexanucleotide signal, AAU AAA, is overlined. The septanucleotide that is homologous to the consensus octanucleotide described by Caput et al. (17) is underlined.

degradation products observed in the S1 mapping and RNase protection experiments (Fig. 5A, lanes 4 to 6, short arrow; see also Fig. 6). Still smaller bands were not detected, suggesting that subsequent degradation steps occurred rapidly.

To determine whether the poly(A) of a stable mRNA was shortened at the same rate as that of unstable c-*myc* mRNA, a blot similar to that shown in Fig. 5A was hybridized with a probe for γ globin mRNA. RNA from unincubated reaction mixtures generated a heterogeneous smear (Fig. 7, lane 1). Thus, the population of γ globin mRNA molecules, like c-*myc* mRNA, contained heterogeneous poly(A) tracts (compare lanes 1 and 8). In contrast to c-*myc* poly(A) tracts, however, most γ globin poly(A) tracts were not excised during the incubation (lanes 1 to 7). Therefore, poly(A) stability correlated with mRNA stability in this instance.

Poly(A) shortening precedes degradation in the 3′ untranslated region. Two observations suggested that poly(A) shortening preceded degradation of the 3′ untranslated region. First, there was a lag before the onset of 3′-region degradation. Second, poly(A) shortening was observed sooner than 3′-region degradation. In an effort to confirm these observations by using a different approach, the degradation of polyadenylated, ^{32}P-labeled c-*myc* and β globin mRNAs was investigated. Each substrate, transcribed with SP6 RNA polymerase and containing a homogeneous poly(A) tract of approximately 85 nt, was incubated for various times in cell-free reaction mixtures. Total RNA was purified and annealed to the c-*myc* antisense 21-mer. Hybrids were treated with RNase H, and reaction products were analyzed by gel electrophoresis. Unincubated c-*myc* mRNA generated the expected fragment, containing 400 nt from the 3′ untranslated region plus 85 nt of poly(A) (Fig. 8A, lane 1). [The oligo(dT)-plus-RNase H control illustrates the difference between the polyadenylated and deadenylated substrates (compare lanes 1 and 8).] Poly(A) shortening occurred throughout the 8-h incubation, albeit at a rate slower than that observed with polysome-bound c-*myc* mRNA. Shortening continued until approximately 30 to 40 A residues were left (lanes 4 to 7), but smaller molecules generated by additional poly(A) shortening or 3′-region degradation were not detected. Rather, the intensity of the oligoadenylated molecules simply diminished with time (compare lanes 4 and 7; see Discussion). This observation suggests that subsequent destruction of the oligoadenylated ^{32}P-labeled c-*myc* mRNA is so rapid that degradation products are too short-lived to be detected. As expected, the β globin poly(A)

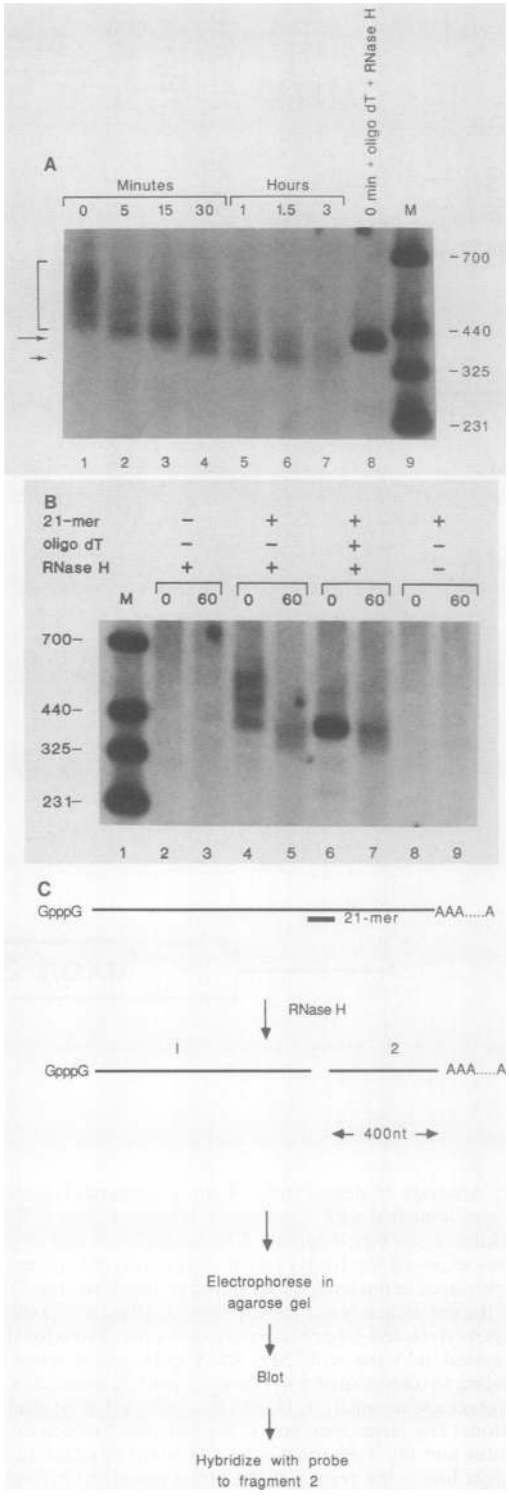

FIG. 5. Analysis of poly(A) shortening and 3′ degradation of c-*myc* mRNA by RNase H-blotting procedure. (A) RNase H blotting of c-*myc* mRNA. RNA (10 μg) from the cell-free reactions shown in Fig. 3 was annealed with a 21-nt, single-stranded DNA complementary to a sequence located 400 nt 5′ of c-*myc* poly(A) site 2 (see panel C and the text). Hybrids were treated with RNase H, and the RNA fragments were electrophoresed and transferred to a Zeta-Probe membrane (Bio-Rad). The blot was hybridized to a [^{32}P]RNA probe complementary to the 3′-terminal 400 nt of c-*myc* mRNA and exposed for 5 days with an intensifying screen. Lanes 1 to 7, RNAs from cell-free reaction mixtures incubated for the indicated times;

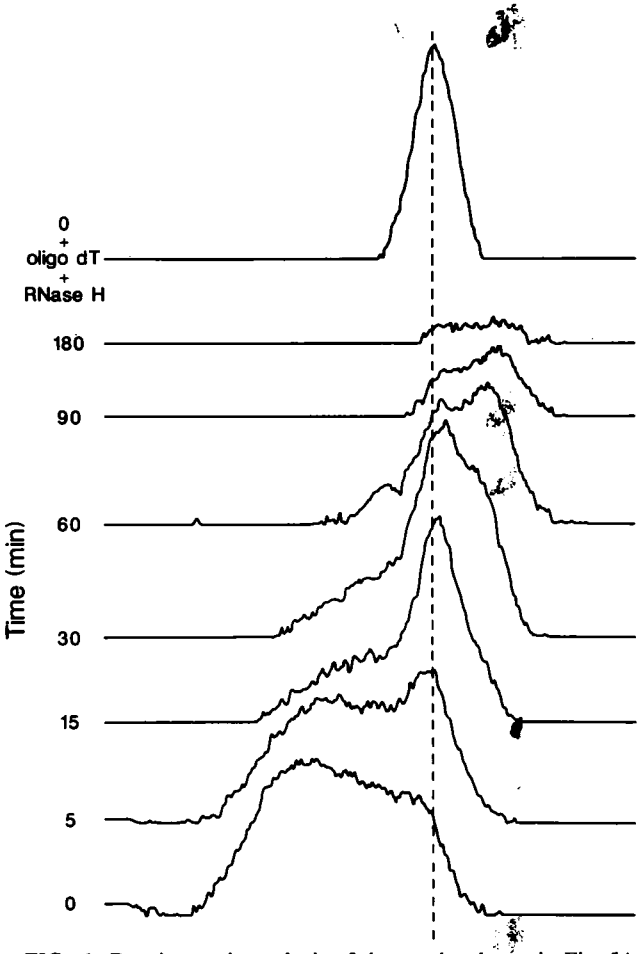

FIG. 6. Densitometric analysis of the results shown in Fig. 5A. The blot was exposed to X-ray film for 2 weeks without a screen, and each lane was scanned with a soft laser densitometer. The bottom seven tracings are for reaction mixtures incubated for the indicated times. The top tracing (0 + oligo(dT) + RNase H) is from Fig. 5A, lane 8, in which RNA from an unincubated reaction mixture was annealed with the 21-mer and oligo(dT) before RNase H treatment (deadenylated c-*myc* mRNA). The relative position of the deadenylated mRNA band with respect to the bottom seven lanes was determined by aligning each tracing with reference to an internal control band that was present at the same location in each lane.

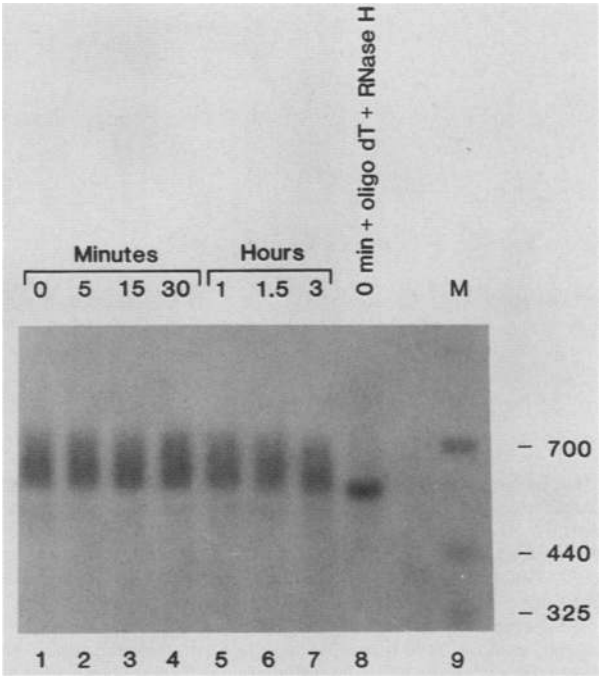

FIG. 7. Stability of γ globin poly(A). A blot similar to the one shown in Fig. 5A was hybridized with a [^{32}P]cDNA probe complementary to human γ globin mRNA and exposed for 2 days with an intensifying screen. Lanes 1 to 7, RNAs from cell-free reaction mixtures incubated for the indicated times; lane 8, RNA from an unincubated reaction mixtured annealed with oligo(dT)12–18 and then treated with RNase H; lane 9, RNA markers as described in the legend to Fig. 5.

lane 8, RNA from an unincubated reaction mixture annealed with both the 21-mer and oligo(dT)12–18 and then treated with RNase H; lane 9, markers (uniformly labeled [^{32}P]RNAs of the indicated sizes (nucleotides), synthesized in vitro with SP6 RNA polymerase from pSP65 DNA cleaved with different enzymes). The bracket indicates unincubated c-*myc* mRNA with its heterogeneous poly(A) tracts. The long arrow indicates deadenylated c-*myc* mRNA (lane 8); the short arrow indicates RNAs corresponding in size to c-*myc* mRNA degradation products. (B) Controls. Lane 1, Markers (as in panel A, lane 9); lanes 2 to 9, 10 μg of RNA from cell-free reaction mixtures incubated for either 0 or 60 min was treated as noted at the top and then electrophoresed, transferred, hybridized, and autoradiographed as described for panel A. (C) Outline of the RNase H blotting procedure.

tract was relatively stable throughout the incubation (Fig. 8B).

This experiment confirmed that poly(A) shortening precedes degradation of the mRNA body. The gradual size reduction of the mRNA is not compatible with single-step endonucleolytic cleavage but is consistent with stepwise poly(A) shortening, as observed in whole cells (14, 59, 89, 90). The shortening reaction does not appear to generate a stable pool of fully deadenylated molecules (Fig. 5 and 8).

Relative stability of the c-*myc* mRNA 5′ region. To determine whether the c-*myc* mRNA 5′ region was degraded as rapidly as its 3′ region, RNAs from in vitro reactions were analyzed by S1 mapping with a 5′ ^{32}P-labeled DNA probe from exon 1. Two major bands were observed with total RNA from K562 cells and from unincubated reaction mixtures (Fig. 9, lanes 2 and 3, arrows). They corresponded to c-*myc* mRNAs with different cap sites, P1 and P2. The intensity of each band did not diminish significantly during the 5-h incubation (lanes 3 to 7). Therefore, the 5′ region was significantly more stable than the 3′ region in vitro. Several S1 nuclease mapping experiments were performed with different probes from exons 1 and 2 in an effort to map the 3′ termini of the stable fragment(s), but no discrete fragments were detected, indicating that the 3′ termini were heterogeneous (data not shown). Sunitha and Slobin (94) also observed that eEF-Tu mRNA was incompletely degraded in their cell-free mRNA decay system and that a stable 5′-region fragment accumulated during the incubation.

DISCUSSION

Our experiments focused on the early steps in c-*myc* mRNA degradation, which occur at its 3′ end. The results

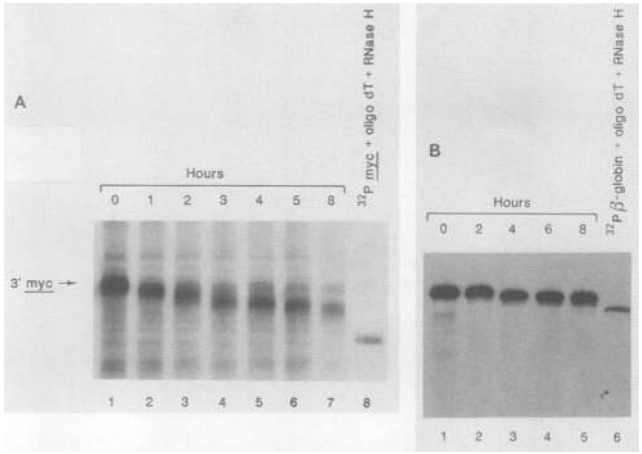

FIG. 8. Shortening of the poly(A) of ^{32}P-labeled c-*myc* mRNA. Separate cell-free decay reaction mixtures containing 60,000 cpm of uniformly ^{32}P-labeled, polyadenylated c-*myc* or β globin mRNA were incubated for the indicated times, and total RNA was extracted. Reaction mixtures containing c-*myc* mRNA were annealed to the c-*myc* antisense 21-mer, as described in the legend to Fig. 5. Hybrids were treated with RNase H, and RNA fragments were analyzed in an 8.3 M urea–3% polyacrylamide gel, which was exposed for 1.5 days with an intensifying screen. Note that the c-*myc* mRNA was polyadenylated at the most distal poly(A) addition site, which is located at an *Afl*II restriction site (see Fig. 4 and the text). (A) 3′ c-*myc* mRNA. Lanes 1 to 7, ^{32}P-labeled c-*myc* mRNA from cell-free decay reaction mixtures incubated for the indicated times and processed as described above; lane 8, ^{32}P-labeled mRNA from an unincubated reaction mixture, annealed with both oligo(dT)12–18 and the 21-mer and then treated with RNase H (deadenylated c-*myc* mRNA). (B) β globin mRNA. Lanes 1 to 5, ^{32}P-labeled β globin mRNA from cell-free decay reaction mixtures incubated for the indicated times; lane 6, ^{32}P-labeled mRNA from an unincubated reaction mixture, annealed with oligo(dT) and treated with RNase H (deadenylated β globin mRNA).

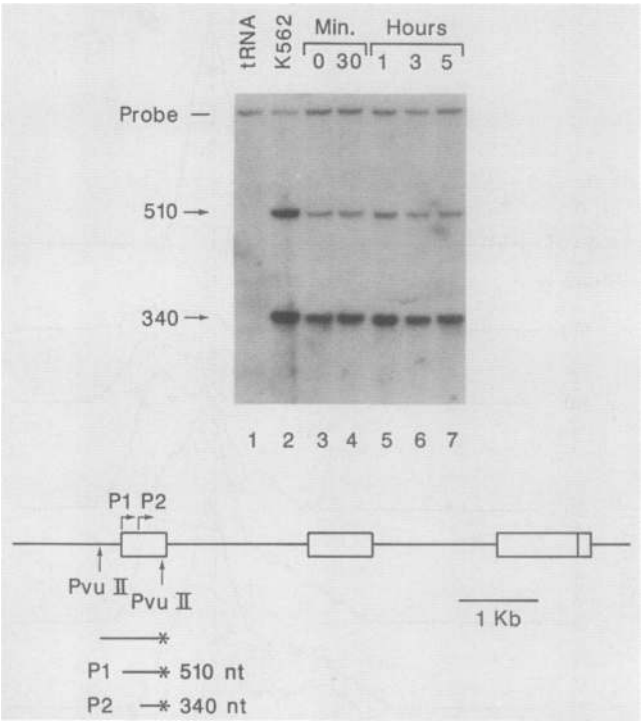

FIG. 9. Analysis of the stability of the 5′ c-*myc* mRNA region by S1 nuclease mapping. Five micrograms each of the same RNAs used in the experiment for which results are shown in Fig. 2 was analyzed by S1 mapping with a 5′ ^{32}P-labeled 800-nt DNA probe complementary to most of the c-*myc* exon 1 sequences. S1-protected fragments were analyzed in a 3% polyacrylamide gel. Autoradiography was performed for 4 days with an intensifying screen. Lane 1, 5 μg of *E. coli* tRNA; lane 2, 5 μg of total RNA from K562 cells; lanes 3 to 7, RNA from cell-free reaction mixtures incubated for the indicated times. The numbers (sizes in nucleotides) on the left indicate the full-length protected fragments, corresponding to cap sites P1 and P2. A diagram of the c-*myc* gene with its three exons (boxes) and two intervening sequences (lines) is shown at the bottom. The symbols are defined in the legend to Fig. 2.

suggest a common feature in the turnover of c-*myc*, H4 histone, eEF-Tu, and probably many other mRNAs as well, namely, degradation in a 3′-to-5′ direction (80, 94). The first step in c-*myc* mRNA decay appears to be poly(A) shortening. The second step, involving cleavage within the oligoadenylated mRNA body, generates discrete degradation products lacking 10 to 30 nt from the 3′ untranslated region. We do not know what types of nucleases are responsible for either step.

Perhaps the major questions raised by our experiments concern the link between poly(A) removal and mRNA degradation. In the two instances we investigated, there is a correlation between poly(A) shortening and degradation. c-*myc* mRNA is unstable and loses its poly(A) before other segments of the molecule are destroyed; γ globin mRNA is stable and is not rapidly deadenylated. We observed only minimal degradation of γ globin mRNA in reaction mixtures incubated for 10 h and, therefore, cannot say whether it is deadenylated before being degraded. A report by Swartwout et al. (95) suggested the possibility that poly(A)$^+$ c-*myc* mRNA was degraded faster than poly(A)$^-$ c-*myc* mRNA in HL-60 cells exposed briefly to actinomycin D. However, recent experiments involving longer actinomycin treatment times indicate that the poly(A)$^+$ mRNA is first deadenylated, generating a pool of poly(A)-deficient c-*myc* mRNA that is subsequently degraded (S. Swartwout and A. Kinniburgh, personal communication). Therefore, the whole-cell experiments of Kinniburgh and colleagues are consistent with our in vitro data.

Although there is some controversy (for a review, see reference 68; 55), most published evidence supports the idea that poly(A) excision precedes mRNA degradation in mammalian cells. First, poly(A) tracts shorten with time (14, 43, 56, 59, 89), and some mRNAs do lose their poly(A) before they are degraded (23, 48, 58, 62, 79, 90, 104, 105; S. Swartwout and A. Kinniburgh, unpublished observations). Second, the addition of poly(A) to some deadenylated mRNAs stabilizes them (29, 41, 42, 70, 93). Third, certain hormones and sugars might stabilize targeted mRNAs by a mechanism involving poly(A) elongation (61, 67, 101). Deadenylation might play a different role in submammalian organisms, for example, affecting translation rates (55, 64, 68, 91).

If poly(A) removal is a prerequisite for mRNA degradation, it becomes important to understand what determines the poly(A) shortening rate. Why is the poly(A) of an unstable mRNA destroyed faster than that of a stable mRNA, if all poly(A) tracts are identical in sequence and, at least initially, similar in size? There are several possible explanations, one of which is that poly(A) is degraded rapidly when it is no longer protected from nucleases by the poly(A)-binding protein (PABP; 11). PABP binds to poly(A) with

high affinity (5, 13, 49) but can also bind to heteropolymeric regions in mRNA (see below). We suggest that such regions are especially prevalent within some unstable mRNAs and that PABP can migrate off of the poly(A) and into these regions. PABP migration could convert the nuclease-resistant poly(A)-PABP ribonucleoprotein complex into protein-depleted, nuclease-sensitive poly(A). Recent experiments with the *Saccharomyces cerevisiae* PABP support this notion (1, 5, 84, 85), because yeast PABP can bind to the 5′ untranslated region of its own mRNA (84). This region is A-rich but also contains other bases. The c-*myc* mRNA 3′ region might function similarly, i.e., as a "sink" for PABP, because it is particularly A+U-rich. (Of the 3′-terminal 200 residues, 75 are A and 82 are U, i.e., 80% A + U.) It also contains short, A-rich clusters, similar to the yeast internal PABP binding site (1, 84).

The apparent length limit of 30 to 40 A residues in c-*myc* mRNA undergoing degradation (Fig. 8) also supports the idea that the poly(A)-PABP complex protects mRNAs from nucleases. A single PABP interacts with approximately 25 to 30 A residues (5, 85), and the stability of globin mRNA with 32 or fewer 3′-terminal A's is equivalent to that of its deadenylated counterpart (66). These observations suggest that poly(A) can be shortened to a minimum length which is still capable of protecting an mRNA (30 to 40 residues, complexed with a single PABP). The minimum is determined by the interaction of PABP with poly(A). If the PABP then dissociates from the minimal complex and the poly(A) is further shortened, it would be impossible for PABP to rebind, and the mRNA would be exposed to rapid nuclease attack. If the mRNA turnover rate is proportional to the poly(A) shortening rate, which, in turn, is a function of internal mRNA sequences, then factors that regulate mRNA turnover could function by interacting with the internal sequences.

The internal sequences that play major roles in mRNA turnover are probably located in the 3′ untranslated region. This region contains some of the signals that determine mRNA degradation rates (17, 20, 75, 83, 88, 97). It is also where mRNA bodies are first attacked (80, 82; this report), and it is highly susceptible to cleavage by exogenous nucleases (11, 86). However, other regions also affect turnover. For example, c-*myc* mRNA lacking exon 1 but containing the intact 3′ region is three- to fivefold more stable than unmodified c-*myc* mRNA (31, 71, 73, 78), and c-*myc* mRNA transcribed from an upstream cap site, P0, is more stable than that transcribed from cap sites P1 or P2 (9). Therefore, nucleolytic attack may occur first in the 3′ region, but sequences in the 5′ region can affect degradation. Similar mechanisms apply to some procaryotic mRNAs. *Escherichia coli ompA* mRNA is degraded 3′ to 5′, but its turnover is determined largely by 5′-region sequences (8, 98).

The stable c-*myc* mRNA fragments that accumulate in vitro include exon 1 sequences and have heterogeneous 3′ termini (Fig. 9 and data not shown). Such fragments might arise via transcriptional attenuation in cells (10, 31, 65). We suggest that degradation is incomplete in vitro because the reactions do not remain active for a sufficient time. Since degradation is asynchronous, it is difficult to measure the number of nucleotides per hour excised from the mRNA. However, the estimated excision rate is 200 nt per h, so it would take 10 to 12 h to degrade c-*myc* mRNA completely. Furthermore, the reaction rate is slower after 3 to 5 h than during hour 1 (Fig. 2, compare lanes 8 and 9 with 4 through 6). An alternative interpretation for the stable 5′ fragments invokes the formation of a stable duplex between segments of exons 1 and 2 (86). The duplex region could protect upstream sequences from nuclease attack.

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