Evidence for a 3′-5′ Decay Pathway for c-myc mRNA in Mammalian Cells*

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Many mRNAs in mammalian cells decay via a sequential pathway involving rapid conversion of polyadenylated molecules to a poly(A)-deficient state followed by rapid degradation of the poly(A)-deficient molecules. However, the rapidity of this latter step(s) has precluded further analyses of the decay pathways involved. Decay intermediates derived from degradation of poly(A)-deficient molecules could offer clues regarding decay pathways, but these intermediates have not been readily detected. Cell-free mRNA decay systems have proven useful in analyses of decay pathways because decay intermediates are rather stable in vitro. Cell-free systems indicate that many mRNAs decay by a sequential 3′-5′ pathway because 3′-terminal decay intermediates form following deadenylation. However, if 3′-terminal, in vitro decay intermediates reflect a biologically significant aspect of mRNA turnover, then similar intermediates should be present in cells. Here, I have compared the in vivo and in vitro decay of mRNA encoded by the c-myc proto-oncogene. Its decay both in vitro and in vivo occurs by rapid removal of the poly(A) tract and generation of a 3′-terminal decay intermediate. These data strongly suggest that a 3′-5′ pathway contributes to turnover of c-myc mRNA in cells. It is likely that 3′-5′ decay represents a major turnover pathway in mammalian cells.

The steady-state levels of mRNAs depend upon their combined rates of synthesis and processing in the nucleus, transport from the nucleus to cytoplasm, and decay in the cytoplasm. An early step in the cytoplasmic decay of many mRNAs in eukaryotes is exoribonucleolytic shortening of the poly(A) tail (reviewed in Refs. 1–4). In the yeast Saccharomyces cerevisiae, the 5′-3′ and 3′-5′ pathways appear to be the two major pathways for mRNA decay. In both pathways, a poly(A) nuclease shortens the poly(A) tract to 10–15 nt.1 In the 5′-3′ pathway, the enzyme Dcp1p then removes the 5′ cap structure, and the Xrn1p exoribonuclease degrades the mRNA 5′-3′ (2, 5–7). In the 3′-5′ pathway, decay of the 10-nt poly(A) tract continues after the initial phase of deadenylation. Degradation within the 3′-UTR then ensues 3′-5′ via the exosome, a heteropentameric protein complex (6, 7). Yeast mRNAs also decay by alternate pathways under some circumstances. For example, some mRNAs decay by an endonucleolytic cleavage event that is the rate-limiting step in their turnover because the cleavage is independent of poly(A) shortening (2). Additionally, mRNAs containing nonsense codons shortening 2 by a pathway that involves deadenylation-independent decay processes (8, 9).

By contrast, decay pathways in mammalian cells are not as well understood. Like yeast mRNAs, many mammalian mRNAs decay initially by exoribonucleolytic shortening of the poly(A) tract. However, subsequent decay steps involving the mRNA body generally occur so quickly that decay intermediates are not detected, thus precluding further analyses of decay pathways. Nonetheless, some recent indirect evidence suggests that mammalian mRNAs may decay by a 5′-3′ pathway. For example, a polymerase chain reaction-based analysis involving simultaneous comparisons of poly(A) tract lengths and presence of a cap structure revealed that only mRNAs with short poly(A) tracts lacked a cap structure (10). This suggests that decapping of mammalian mRNAs occurs once deadenylation activities shorten the poly(A) tract to some critical length. Additional evidence for a 3′-5′ decay pathway in mammalian cells is the identification of a murine XRN1 homologue that functions in S. cerevisiae (11). Together, these data suggest that 5′-3′ mRNA decay processes may be conserved in eukaryotes.

A 3′ to 5′ decay pathway for mammalian, polyadenylated mRNAs is also likely for several reasons. (i) HeLa cells, a human cervical carcinoma cell line, contain a complex homologous to the yeast exosome (6). (ii) Human H4 histone mRNA, which lacks a poly(A) tract, decays 3′-5′ in cells (12). Degradation is likely because of an exoribonuclease that pauses within the 3′-terminal stem-loop structure, generating progressively shorter decay intermediates that lack 5 nt, then 12 nt, from the 3′ end (13). (iii) In vitro mRNA decay extracts prepared from mammalian cells degrade labile mRNAs 3′-5′ by rapid deadenylation followed by generation of 3′-terminal decay intermediates (reviewed in Refs. 14 and 15). The same enzymes operative in vivo presumably generate these intermediates in vitro. However, detection of such mRNA decay intermediates in cells has proven difficult (1, 14). Thus, an unanswered question is whether 3′-5′ decay of polyadenylated mRNAs occurs in mammalian cells as indicated by 3′-terminal decay intermediates. Here, I have addressed this question by comparing the decay of c-myc mRNA in vitro and in cells. c-myc mRNA, which encodes the Myc transcription factor and oncoprotein, is a labile, polyadenylated transcript (reviewed in Ref. 16). In vitro, it rapidly decays by a sequential pathway involving rapid deadenylation to a deadenylated or oligoadenylated form; this is followed by degradation of the mRNA body generating easily observed 3′-terminal decay intermediates. Its decay then continues in a 3′-5′ direction (17). c-myc mRNA decays by rapid deadenylation in vivo as well. Also observed is a 3′-terminal decay intermediate that appears to have a similar 3′ end as an intermediate generated during in vitro decay. These data provide strong

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1 The abbreviations used are: nt, nucleotide(s); RNase, ribonuclease; UTR, untranslated region.
evidence that a 3′-5′ pathway contributes to decay of c-myc mRNA, and perhaps other polyadenylated mRNAs, in mammalian cells.

EXPERIMENTAL PROCEDURES

Restriction enzymes and RNAses were obtained from Promega Corp. (Madison, WI). RNase H, oligo(dT)12-18, and oligo(dT)-cellulose were from Amersham Pharmacia Biotech. Creatine phosphate, creatine phosphokinase, and actinomycin D were from Calbiochem (La Jolla, CA). [α-32P]UTP and [α-32P]dCTP were from ICN Biomedicals (Irvine, CA). Oligodeoxynucleotide synthesis was performed by Operon Technologies (Alameda, CA). All other reagents were molecular biology grade.

Actinomycin D Treatment of Cells, Preparation of RNA, and RNase H Analysis—Exponentially growing K562 cells, a human erythroblastic leukemia-like cell line (18, 19), were cultured with 5 μg/ml actinomycin D for various lengths of time at 37 °C. For each time point, cells were harvested and total RNA was prepared by lysis of cells, phenol extraction of proteins, and pelleting of RNA through a pad of CsCl as described (12). RNA concentrations were determined spectrophotometrically by absorption at 260 nm. Ten micrograms of each RNA sample were subjected to oligonucleotide-directed RNase H cleavage using an antisense c-myc oligodeoxynucleotide (5′-CAAGTGATAGGGCTGCTG-3′) which anneals approximately 400 nt upstream of poly(A) site 2 (17). Deadenylated RNAs were prepared in vitro by incubating the time-zero RNA sample with oligo(dT)12-18 and RNase H. The RNA samples were fractionated in a denaturing agarose gel and blotted to a membrane. The γ-globin mRNA or the 3′-end of c-myc mRNA was detected by incubating the membrane with the respective 32P-labeled probes, washing the membrane, and exposing it to x-ray film as described (17). This RNase H mapping procedure permits high resolution analysis of cleavage products at the 3′ end of c-myc mRNA (20, 21).

Oligo(dT)-cellulose Chromatography of Cellular RNA—Ten milligrams of RNA purified from 5 × 10⁶ exponentially growing K562 cells was heat-denatured and loaded onto a column containing 400 μl of oligo(dT)-cellulose in column binding buffer (10 mM Tris-HCl, pH 8.1, 0.5 mM GTP, 0.1 mM spermine, and 40 units of RNasin). Some reactions were supplemented with 300 μg of S130 proteins. RNA was purified for each time point, and c-myc mRNA was analyzed by an RNase P1+T1 protection assay as described above.

RESULTS

Cell-free mRNA decay systems degrade mRNAs via a 3′-5′ pathway involving gradual removal of the poly(A) tract followed by 3′-terminal degradation of the mRNA body. To examine the decay pathway of c-myc mRNA in cells, exponentially growing K562 cells were cultured in the presence of actinomycin D to inhibit new transcription. At various time points, cells were harvested for purification of total RNA. Decay of c-myc mRNA was investigated by a modified Northern blot procedure that involves oligonucleotide-directed RNase H cleavage of c-myc mRNA in purified RNA samples, prior to the gel electrophoresis and blotting steps. c-myc mRNA is cleaved approximately 400 nt from its 3′ end by this procedure. Thus, the RNase H mapping assay permits examination of 3′-end decay at a resolution higher than that allowed by traditional Northern blotting (17, 20, 21). Fig. 1 shows the results of this analysis. The time-zero point showed a heterogeneous smear (Fig. 1, top panel, lane 1). Additional treatment of the time-zero RNA with oligo(dT)12-18 and RNase H reduced this smear to a single band (top panel, lane 8). This indicated that the smear was because of heterogeneous lengths of poly(A) tails within the population of cellular c-myc mRNA molecules. With increasing time following inhibition of transcription, the poly(A) tails overall became shorter. By 90 min, most c-myc mRNA molecules were completely deadenylated or had short adenylate tracts (Fig. 1, top panel; compare lane 6 with lane 8). Rapid deadenylation of c-myc mRNA was specific because the poly(A) tract of γ-globin was not rapidly shortened, and the mRNA was stable over the 3-h time course (Fig. 1, lower panel). Thus, c-myc mRNA was rapidly deadenylated in cells generating deadenylated or oligoadenylated mRNA molecules. These results are consistent with those from earlier studies showing rapid deadenylation of c-myc mRNA in cell-free decay reactions (17).
FIG. 2. Detection of 3'-terminal decay intermediates in vivo. A, the same RNA samples utilized in Fig. 1 were analyzed by an RNase P1 + T1 protection assay using a 620-nt, 32P-labeled probe spanning the last 210 nt of c-myc mRNA as described under “Experimental Procedures.” Protected fragments were separated in a 6% polyacrylamide, 7 M urea gel and detected by a 16-h exposure to x-ray film. pA2 indicates the protected fragments corresponding to poly(A) site 2 molecules (17). The arrow denotes the location of a protected fragment corresponding to a likely decay intermediate (see text). Lane M contains 32P-labeled DNA markers with nucleotide lengths denoted to the right of the panel. B, the gel was re-exposed to x-ray film for 14 days to enhance the signal for the decay intermediate (denoted by the arrow). A diagram depicting the 3' end of the human c-myc gene is shown at the bottom. The 3'-UTR and 3'-flanking genomic DNA are denoted by the box and line, respectively. The two major polyadenylation sites are shown along with the SspI and EcoRI sites used for generation of the probe. The short line represents the region of the probe protected by undegraded c-myc mRNA that terminates at poly(A) site 2. C, RNase protection control experiment. Plasmid pGB1023 contains the full-length cDNA encoding the human c-myc mRNA that ends at the 3'-most polyadenylation site (17). Transcription of the HindIII-digested plasmid with SP6 RNA polymerase produces full-length mRNA with an 85-nt poly(A) tract. RNA from K562 cells (lane 2) and c-myc mRNA synthesized in vitro (lane 3) were analyzed by the RNase P1 + T1 protection assay as described in panel A. Samples were fractionated in the same 6% polyacrylamide, 7 M urea gel, and protected fragments were detected by autoradiography. Lanes 1 and 2 were exposed for 5 days while lane 3 was exposed for 16 h. The arrow in lane 2 denotes the band corresponding to the decay intermediate.

FIG. 3. Assay of the 3'-terminal, c-myc decay intermediate in poly(A2) versus poly(A1) RNA fractions. Total RNA from exponentially growing K562 cells was fractionated by oligo(dT)-cellulose chromatography as described under “Experimental Procedures.” A, one microgram of the indicated RNAs was fractionated in a miniature, 1% agarose gel and visualized by ethidium bromide staining. The locations of the 28 and 18 S ribosomal RNAs are denoted to the left of the panel. B, equal cell equivalents of total RNA, poly(A2) RNA, and poly(A1) RNA were analyzed for H4 histone or γ-globin mRNAs by nuclease S1 mapping as described under “Experimental Procedures” using 32P-labeled probes for each mRNA. Incubation of probes with tRNA served as hybridization controls (lanes 2 and 6). The protected fragments were fractionated on the same 8% polyacrylamide, 7 M urea gel, detected by autoradiography, and quantified by densitometry. The protected fragment lengths are 104 nt for H4 histone and 167 nt for γ-globin mRNA. The sizes of 32P-labeled DNA marker fragments are indicted to the left of the panel. C, equal cell equivalents of total RNA, poly(A2) RNA, and poly(A1) RNA were analyzed for c-myc mRNA by RNase P1 + T1 protection assay using a 32P-labeled probe spanning the last 210 nt of c-myc mRNA as described in the legend for Fig. 2. pA2 indicates the protected fragments corresponding to poly(A) site 2 molecules. Lane M contains 32P-labeled DNA markers with lengths indicated in nucleotides. The gel was exposed to x-ray film for 16 h. D, the gel in panel C was re-exposed to x-ray film for 14 days to enhance the signal for the decay intermediate detected in the poly(A1) fraction (denoted by the short arrow in lane 3).

formation of abundant decay products mapping to the 3'-UTR (17). By contrast, none were obvious in the RNA blot analysis of in vivo decay shown in Fig. 1. However, it was likely that any putative 3'-terminal decay products would be very unstable in cells and thus be low in abundance (1, 14). Therefore, the RNA samples utilized in Fig. 1 were analyzed for possible 3'-termi-
nal decay products by a sensitive RNase P1+T1 protection assay. A 620-nt radiolabeled RNA probe complementary to the 3'-terminal 210 nt of c-myc mRNA was employed for this assay (Fig. 2A). The time-zero RNA generated four protected fragments corresponding to intact c-myc mRNA molecules polyadenylated at four closely spaced sites, referred to as poly(A) site 2 (17). These bands diminished in intensity at approximately equivalent rates during the time course. Quantitation of band intensities as a function of time indicated a half-life of 45 min for c-myc mRNA. Also observed was a very faint protected fragment that was shorter than the four full-length c-myc mRNA species; it also decreased in intensity with time (Fig. 2A, arrow). A 14-day overexposure of the gel enhanced its intensity especially at later time points (Fig. 2B, lane 1). This band was not an artifact of the RNase-digested probe because the control incubation of probe with tRNA did not produce a band at this location in the gel (Fig. 2B, lane 1). Additionally, this band was not detected in a hybridization of the probe with full-length, in vitro synthesized c-myc mRNA, even though the protected fragment corresponding to full-length mRNA was over-exposed (Fig. 2C, compare lane 3 to lane 2). These data suggested that the faint, protected fragment corresponded to a low abundance, 3'-terminal product of c-myc mRNA decay in cells.

If this band represents a decay intermediate generated following deadenylation, then it should be detectable in a poly(A⁻) fraction of RNA from K562 cells and not be detectable in a poly(A⁺) fraction. RNA was fractionated by chromatography using oligo(dT)-cellulose (see “Experimental Procedures”). Analysis of the RNA samples by agarose gel electrophoresis indicated efficient separation of ribosomal RNA (Fig. 3A). As a further control for the separation of poly(A⁻) and poly(A⁺) RNAs, fractions were analyzed for the distribution of H4 histone mRNA, which lacks a poly(A) tract; γ-globin mRNA, which is a polyadenylated mRNA with a half-life of 20 h; and c-myc mRNA, which is a labile, polyadenylated mRNA. Levels of H4 histone and γ-globin mRNAs were measured by nuclease S1 mapping assays. Quantitation of the protected fragments and comparison of signals to the signal present in total RNA indicated that all of the H4 histone mRNA was detected in the poly(A⁺) fraction; none was detected in the poly(A⁻) fraction (Fig. 3B, lanes 3–5). As expected, most (80%) of the γ-globin mRNA was present in the poly(A⁺) fraction (Fig. 3B, lanes 7–9). These analyses indicated a suitable separation of poly(A⁻) and poly(A⁺) RNAs by oligo(dT)-cellulose chromatography. The distribution of c-myc mRNA was examined by RNase P1+T1 protection assay of the fractionated RNAs. Sixty percent of c-myc mRNA was present in the poly(A⁺) fraction and 40% was in the poly(A⁻) fraction (Fig. 3C), indicating that a substantial fraction of the full-length mRNA is likely deadenylated. A 14-day overexposure of the gel showed the presence of the band corresponding to the decay intermediate in the poly(A⁻) fraction while none was detectable in the poly(A⁺) fraction (Fig. 3D, compare lane 3, arrow with lane 4). These data further suggested that the faint, protected fragment observed in RNase protection assays of cellular RNA is a decay intermediate of c-myc mRNA.

Additionally, the results of the cellular decay experiments were compared with those from cell-free decay reactions, where decay products are readily observed (17). RNase P1+T1 protection analysis of c-myc mRNA in decay reactions containing polysomes revealed a major protected fragment with a size similar to the one observed during in vitro decay (Fig. 4A, band I). (The faint, minor band above product I is not consistently observed.) A smaller, less abundant fragment not readily detected in cellular RNA was also observed (Fig. 4A, band II). Bands I and II increased in abundance during incubation times up to 60 min, after which they decreased in abundance. These results were consistent with their being transient products of 3'-terminal decay in vitro. Decay product I was also present in a trace amount in time-zero RNA. This was not unexpected because c-myc mRNA molecules should be undergoing decay in K562 cells at the time of cell lysis. As shown in earlier studies,
3'-terminal decay products form in vitro following removal of most, if not all, of the poly(A) tract (17). Like the 3'-terminal decay product in cells, in vitro decay products also fractionate with poly(A)– RNA by oligo(dT)-cellulose chromatography.2

The stabilities of some mRNAs, such as c-myc, are regulated by cytosolic factors, such as the S130 fraction, not associated with polysomes (23). Thus, formation of 3'-terminal decay intermediates was compared in cell-free reactions containing polysomes alone versus reactions containing polysomes and S130 proteins. In the S130-supplemented reactions, the abundance of decay product I was reduced compared with reactions lacking S130 (Fig. 4B, compare lanes 2–8 with lanes 9–11). This result was consistent with the ability of S130 components to accelerate 3'-terminal decay of c-myc mRNA (23, 24). Moreover, the low abundance of decay product I in S130-supplemented reactions reflected the low abundance of the 3'-terminal decay intermediate observed in cells (compare Fig. 4B, lanes 2–8 to Fig. 2). Decay product II, observed in cell-free reactions containing polysomes without S130, was not readily apparent in either S130-supplemented decay reactions (Fig. 4B, compare lane 11 to lanes 2–8) or in vivo (see Fig. 2). This could be because of its low abundance in these two cases. Altogether, comparison of the in vivo data with the in vitro data strongly argues that decay of c-myc mRNA in cells involves rapid deadenylation and generation of a low abundance, 3'-terminal decay intermediate.

Additionally, size comparison of the 3'-terminal decay product in cellular RNA and decay product I in cell-free reactions suggested they were the same (Fig. 4B, compare lane 13 to lanes 2–11). Therefore, an RNase protection assay was performed with RNA prepared from K562 cells and RNA prepared from in vitro decay reactions (with and without added S130 proteins). These were run in a gel side by side with a DNA sequencing ladder. Fig. 5 shows that in each RNA sample, the 3' ends of the decay products mapped within one or two nt of each other near the 3' termini of the four full-length c-myc mRNA species. These results strongly suggest that the 3'-terminal decay product I generated in vitro represents molecules present in cells as well.

**Discussion**

In vitro, c-myc mRNA decays via a sequential pathway involving rapid conversion of polyadenylated mRNA molecules to a deadenylated or perhaps an oligoadenylated form. Subsequently, 3'-terminal degradation within the 3'-UTR occurs (17). Thus, c-myc mRNA clearly decays in vitro by a 3'-5' pathway (see also Ref. 25). If this pathway is biologically significant, then equivalent decay intermediates should be present in cells. The data presented here indicate that decay of c-myc mRNA in cells involves rapid deadenylation to a deadenyalted or perhaps an oligoadenylated form (Fig. 1). A low abundance, 3'-terminal product of c-myc mRNA decay is present in cells (Fig. 2). The product is only observed in a poly(A)– fraction of cellular RNA prepared by oligo(dT)-cellulose chromatography (Fig. 3). This observation strongly suggests that the product is likely a bona fide mRNA decay intermediate rather than a minor cleavage/polyadenylation site. Additional support for this conclusion comes from the finding that a similar 3'-terminal decay product is observed in cell-free mRNA decay reactions (Fig. 4). Finally, both the in vitro and in vivo decay intermediates map to a similar location near the 3' terminus of the 3'-UTR (Fig. 5). Taken together, these data are consistent with the conclusion that a 3'-5' decay pathway contributes to c-myc mRNA turnover in cells.

The mRNA decay intermediates generated in vivo and in

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2 G. Brewer, unpublished observation.
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cells c-myc mRNA decays directly without prior conversion to a
deadenylated form (27). This may involve inactivation of the
c-myc mRNA-binding protein CRD-BP and subsequent un-
masking of an endoribonuclease site within the 3′-coding re-

region (28–30). Additionally, the action of the endoribonuclease
apparently does not require prior removal of the poly(A) tract.
Thus, endoribonucleolytic cleavage of the coding region, rather
than poly(A) shortening, may be the rate-limiting step in c-myc
mRNA decay during differentiation.

Additionally, a 3′-5′ pathway does not exclude a 5′-3′ path-
way acting simultaneously. In the yeast S. cerevisiae, some
mRNAs decay by both 5′-3′ and 3′-5′ pathways (7). It is likely
that c-myc mRNA decays in vivo by both pathways as well. This
is based upon the observation that nuclease protection assays
employing probes specific for either the 5′ or 3′ end indicated
little difference in the measured half-life.2 By contrast, H4
histone mRNA decays predominantly 3′-5′ (12). In any event, a
possible advantage to the cell for having multiple pathways to
degrade mRNAs would be to provide redundancy in the event
one pathway were to become inactivated because of mutation,
for example. In this case, the additional mRNA turnover path-
way(s) could function. Consistent with the necessity of RNA
turnover processes is the demonstration that they are essential
for viability of both yeast and bacteria. For instance, blocking
both 5′-3′ and 3′-5′ decay pathways in yeast leads to inviability
(7). Likewise, abating 3′-exoribonuclease activity in E. coli by
mutation of both polynucleotide phosphorylase and RNase II
leads to inviability (31). Messenger RNA decay processes will
likely be essential for mammalian cells as well.

In summary, I have compared decay of c-myc mRNA in vivo
and in vitro and determined that similar decay intermediates
are generated in both systems. The data are consistent with the
conclusion that a 3′-5′ decay pathway contributes to the turn-
over of c-myc mRNA in vivo. Finally, these data firmly estab-
lish the fidelity of the in vitro mRNA decay system for 3′-5′
decay of polyadenylated mRNAs. Future studies will seek to
identify, purify, and characterize the relevant activities to re-
constitute mRNA decay from purified components.

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