The neoplastic phenotype is often characterized by the uncoupling of the normally opposing processes of cellular proliferation and differentiation (reviewed in Refs. 1–3). The mechanisms that disrupt their normal coupling are believed to involve the overexpression or inappropriate expression of hematopoietic growth factors and cytokines, their receptors, and/or certain protooncogenes. For example, overexpression of the c-myc protooncogene induces the neoplastic transformation of hematopoietic cells in animals and in cultured cells. Likewise, constitutive expression of the c-myc gene inhibits induced differentiation of mouse erythroleukemia cells and human monocytic cells.

The control of mRNA turnover is an important means of regulating both the level and timing of gene expression. Messenger RNAs like c-myc, c-fos, and granulocyte-macrophage colony-stimulating factor mRNA, whose protein products influence proliferation and differentiation, are relatively unstable, with half-lives of 1 h or less (reviewed in Ref. 4). As a result of their instability, modest changes in their turnover rates affect their steady-state levels over a relatively short time period (5). This short-term regulation ensures that the concentrations of these mRNAs are maintained within a limited range. The necessity of this precise regulation is consistent with their inappropriate expression interfering with proliferation and differentiation.

c-myc mRNA can be degraded by alternative pathways in cells. One involves an endonuclease that recognizes a C-terminal coding region determinant or CRD (reviewed in Ref. 6). This endonuclease cleaves the mRNA within the CRD. A CRD-binding protein can also protect the mRNA from endonucleolytic attack. The other decay pathway involves 3′ to 5′ decay where poly(A) removal occurs prior to degradation of the body of the mRNA (reviewed in Ref. 4). A 3′ to 5′ pathway is also observed during decay of mRNAs in the yeast Saccharomyces cerevisiae, suggesting that this decay mechanism may be common between yeast and humans (7, 8).

Dissection of both mRNA decay pathways and the associated protein machinery has benefited greatly from the development of cell-free mRNA decay systems (reviewed in Refs. 9 and 10; see also Ref. 11). From a biochemical point of view, these systems offer several advantages over studies involving intact cells. (i) Decay reaction rates are generally slower in vitro. This allows detection of decay intermediates that are difficult to detect in cells. Decay intermediates can offer important clues as to the nature of the decay pathway and the enzymes involved. (ii) In vitro systems can serve as an assay for the purification of relevant decay activities. (iii) Finally, in vitro systems can facilitate the study of mRNA decay in organisms for which genetic methods are not well developed.

However, the study of c-myc 3′ to 5′ decay in vitro has been hampered by the inability to reconstitute decay of the mRNA using radiolabeled, exogenous RNA substrates. For example, exogenous c-myc mRNA is quite stable, even after 8 h of incubation at 37 °C, when added to a polysome-based mRNA decay system (12). Here I have developed and utilized a novel cell-free system that reconstitutes 3′ to 5′ decay of exogenous mRNAs in a 3′-UTR-dependent fashion. In this system a chimeric mRNA containing the c-myc 3′-UTR, which targets the mRNA for 3′ to 5′ decay in cells (13–15), is rapidly and specifically deadenylated compared with β-globin mRNA, which is deadenylated very slowly. Following rapid removal of the poly(A) tract, decay intermediates with 3′-termini lying within the c-myc 3′-UTR are generated. This system has permitted, for the first time, the examination of biochemical properties of the c-myc 3′ to 5′ decay activities.

**EXPERIMENTAL PROCEDURES**

All restriction enzymes were obtained from Promega Corp. (Madison, WI). RNase H and oligo(dT)12–18 were from Amersham Pharmacia Bio-

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1 The abbreviations used are: CRD, coding region determinant; nt(s), nucleotide(s); PAB, poly(A)-binding protein; PAN, poly(A) nuclease(s); UTR(s), untranslated region(s).
tech. Creatine phosphate and creatine phosphokinase were from Boehringer Mannheim. [\textsuperscript{\textalpha-\textbeta}-\textP]UTP was from ICN Biomedicals (Irvine, CA). The cap analog "mG(5\textprime)ppp(5\textprime)G" was from New England Biolabs (Beverly, MA). Deoxyligounucleotide synthesis was performed by Operon Technologies (Alameda, CA). All other reagents were molecular biology grade.

Preparation of Salt-washed Polysomes and S100—Exponentially growing K562 cells, a human erythroleukemia-like cell line (16), were harvested, washed in phosphate-buffered saline, and lysed by homogenization in low salt buffer A (10 mM Tris-HCl (pH 7.6), 1 mM magnesium acetate, 1.5 mM potassium acetate, 2 mM dithiothreitol, 1 \mu g each of leupeptin and pepstatin A per ml, 0.1 mM phenylmethylsulfonyl fluoride). Nuclei were removed by centrifugation at 500 \times g for 15 min at 4 °C. Polysomes in the postnuclear supernatant were salt-washed in the presence of crude cytoplasm as described by Ross et al. (17). Briefly, 2 M KCl was slowly added to the postnuclear supernatant with constant stirring at 4 °C to a final concentration of 0.15 M KCl. The salt-washed polysomes were pelleted by centrifugation at 100,000 \times g for 1 h at 4 °C, resuspended in Buffer A, and stored at -80 °C. The supernatant (S100) was concentrated by dialysis in Buffer A containing 20% glycerol and stored in aliquots at -80 °C. Protein concentration of the S100 was measured to be 6 mg/ml by Bradford assay (18).

Preparation of Unwashed Polysomes—Unwashed polysomes were prepared from K562 cells using Buffer A exactly as described by Brewer and Ross (19).

Radiolabeling of mRNA Substrates—Capped human \beta-globin mRNA and \beta-globin-c-myc chimeric mRNAs were synthesized by transcription of HindIII-digested plasmids PSpk\beta and Pm\beta-cmyc (gifts of J. Ross), respectively, using SP6 RNA polymerase, "mG(5\textprime)ppp(5\textprime)G" cap analog, and [\textalpha-\textP]UTP (>500 Ci/mmol). mRNAs were labeled to a specific activity of 20,000 cpm/nmol. Both contain an 85-nt poly(A) tract encoded by the plasmids. Plasmid Pm\beta-cAT is a derivative of PSpk\beta in which \beta-globin sequence 3\textprime of the EcoRI site in the coding region was replaced with human c-myc 3\textprime-UTR sequence (positions 5816 to 6003 (Ref. 21)) ending at, and including, polyadenylation site 2.

In Vitro mRNA Decay Reactions and RNase H Mapping—In vitro mRNA decay reactions were incubated at 37 °C in 20-\mu l reactions with 1–2 \mu g radiolabeled \beta-globin or \beta-globin-c-myc chimeric mRNA in a buffer containing 10 mM Tris-HCl (pH 7.6), 5 mM magnesium acetate, 100 mM potassium acetate, 2 mM dithiothreitol, 10 mM creatine phosphate, 1 \mu l of creatine phosphokinase, 1 mM ATP, 0.4 mM GTP, 0.1 mM dithiothreitol, 1 \mu M spermine, and 70 \mu g of S100 extract. No differences in results were observed with or without RNasin (placental ribonuclease inhibitor; data not shown). For each time point RNA was purified and subjected to oligonucleotide-directed RNase H cleavage using either the antisense c-myc deoxyoligonucleotide 5\textprime-GAAACATTGTGAAACTTAAAATT-3\textprime or the antisense \beta-globin deoxyoligonucleotide 5\textprime-GAAATTCAGATGGAGCAATTTT-3\textprime or the antisense \beta-globin deoxyoligonucleotide 5\textprime-GAAATTTGGAAGAAGCAAGCAAAGGCGGAGC-3\textprime as described by Brewer and Ross (19). This separates 210 nt of the c-myc 3\textprime-UTR and poly(A) tract or 107 nt of the human \beta-globin 3\textprime-UTR and poly(A) tract from the remainder of the respective mRNAs thus permitting high resolution analyses of cleavage products. Reactions were terminated at the 3\textprime end of each mRNA (19, 22). Purified RNAs were fractionated in a denaturing agarose gel and detected by autoradiography. Poly(A) \textsuperscript{\textbeta}mRNAs were prepared by incubating the RNAs with oligo(dT)\textsubscript{12-18} and RNase H.

In vitro mRNA decay reactions with polysomes were performed as described above with the inclusion of RNasin. Purified RNA was subjected to RNase H mapping analysis for c-myc mRNA using a deoxyoligonucleotide (5\textprime-CAAGTTCTAGATGGTAAGGCTC-3\textprime ) complementary to a region 400 nt from polyadenylation site 2 (12). RNA was then fractionated on a 2% agarose-formaldehyde gel, blotted to a membrane, incubated with either a \textsuperscript{\textbeta}-P-labeled probe for the c-myc 3\textprime-UTR or \gamma-globin mRNA, washed, and exposed to film (12).

RESULTS

The major decay pathway for c-myc mRNA in exponentially growing cells involves gradual removal of the poly(A) tract followed by degradation of the mRNA body (19–21). Many of the factors involved in these processes have yet to be identified or characterized. To characterize the protein factors involved in these processes, it is necessary that they be solubilized in an active form. To address this I first compared c-myc mRNA decay in vitro using polysomes versus polysomes that were salt-washed to remove (i.e. solubilize) putative factors. A Northern blot analysis of these decay reactions showed that although polysome-bound c-myc mRNA was degraded by deadenylation in unwashed polysomes, it was stable in polysomes washed with KCl (Fig. 1, compare lanes 2–5 with lanes 6–9). This result suggests that washing releases decay activities from polysomes, thus stabilizing the polysome-bound c-myc mRNA. It is important to note two points. (i) Our washing procedure differs from that of Schreier and Staehelin (23) in that our K562 cell polysomes are washed in the presence of crude cytoplasm, which prevents degradation of ribosomal RNAs and mRNAs (17). (ii) The quantity of c-myc mRNA per 260 unit of washed polysomes is greater compared with unwashed polysomes. This is likely because of removal of loosely bound RNAs (and proteins) by washing which would cause apparent increases in c-myc mRNA per washed polysome. (In this regard see also Ref. 17.)

To determine whether in fact c-myc mRNA decay activities could be solubilized, polysomes were salt-washed in the presence of crude cytoplasm. The 100,000 \times g supernatant (S100) from this procedure was used in decay reactions containing either \textsuperscript{\textbeta}-P-labeled \beta-globin mRNA or \beta-globin-c-myc chimeric mRNA substrate. Reactions were also supplemented with poly(A) to effectively deplete the reactions of poly(A)-binding protein (PAB), which indiscriminately inhibits decay of polyadenylated mRNAs in extracts (24, 25). RNA was purified from each time point and then assayed by deoxyoligonucleotide-directed RNase H cleavage of the purified mRNA near the 3\textprime end to permit high resolution analysis of 3\textprime-end decay products by polyacrylamide gel electrophoresis. As shown in Fig. 2A the initial decay event(s) appeared to be deadenylation, which was followed by degradation of the mRNA body generating discrete, 3-end decay products (denoted by arrows in Fig. 2A). Their sizes are consistent with their 3\textprime ends lying within the c-myc 3\textprime-UTR. The use of several oligonucleotides complementary to different regions of the c-myc 3\textprime-UTR in the RNase H mapping procedure confirmed that the 3\textprime ends of the decay products lie within the c-myc 3\textprime-UTR and not in other regions of the mRNA (data not shown). A control experiment indicated that the appearance of these specific bands in the gel required the deoxyoligonucleotide and RNase H (data not shown). Together, these results indicate that deadenylation occurs before decay of the mRNA body in this system. Thus, the decay pathway would

![Fig. 1. In vitro decay of c-myc mRNA in unwashed versus washed polysomes.](http://www.jbc.org/)

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to a marker lane where the nucleotide lengths are denoted on the left of the panel. The blot was stripped and reprobed for γ-globin mRNA (bottom panel).

decay activities can be solubilized by salt treatment, that they can act upon mRNA that is not associated with ribosomes, and that in solubilized form they can reconstitute enzymatic activities exhibited by polysome-bound activities on polysome-bound mRNA. Moreover, these data indicate that the destabilizing potential of the c-myc 3′-UTR can be transferred to a normally stable mRNA within the context of the soluble-based biochemical system, just like it can in vivo (13, 14, 26). Together, these observations suggest that the soluble-based biochemical system performs with the same fidelity as a polysome-based system and can thus reconstitute physiologically relevant mRNA decay processes.

The soluble-based system permitted a number of properties of the c-myc 3′ to 5′ decay activities to be investigated for the first time, and the results are summarized in Table I. Decay was not inhibited by the placental ribonuclease inhibitor RNasin, indicating that the activities are not inhibited by the RNase A class of RNase inhibitors. Decay required divalent cation with optimal concentrations of Mg2+ between 2 and 5 mM. Decay did not require exogenous ATP/GTP or an ATP-regenerating system. The results of the mRNA decay experiments were the same whether or not the mRNAs were capped, and decay activity was not affected by the addition of up to 0.5 mM cap analog 7mG(5′)ppp(5′)G to the reactions. This latter result suggests that the activities of at least some components of the c-myc 3′ to 5′ decay machinery cannot be titrated by a 7mG(5′)ppp(5′)G cap structure.

Because the early step(s) in decay appeared to be deadenylation, the requirement of a poly(A) tract to nucleate the assembly of the 3′-UTR-dependent degradation activity was examined. Chimeric β-globin/c-myc mRNA lacking a poly(A) tract was prepared and used in decay reactions. As shown in Fig. 4, formation of 3′-end decay intermediates (denoted by arrows) was not inhibited. However, given this result, it was somewhat surprising that the kinetics of formation of the decay intermediates was not accelerated with the poly(A−) mRNA compared with the poly(A+) mRNA (compare Fig. 4 with Fig. 2A). In any event, although deadenylation appears to occur before degra-
TABLE I

Biochemical properties of 3′ to 5′ decay activities

| RNase inhibitor       | 0–1 mM Mg2+ | 2–5 mM Mg2+ | ATP/GTP | None added | 1/0.4 mM Cap analog | None added | 0.1–0.5 mM Cap analog |
|-----------------------|-------------|-------------|---------|------------|---------------------|------------|-----------------------|
| None added            | Active      | Active      | Active  | Active     | Active              | Active     | Active                |
| RNasin                | Active      | Active      | Active  | Active     | Active              | Active     | Active                |
| Mg2+                  | Inactive    | Inactive    | Inactive Active | Inactive    | Inactive            | Inactive    | Inactive             |

*a The terms active and inactive refer to the decay rates of the β-globin-c-myc chimeric mRNA as compared to the decay rates under standard reaction conditions (see “Experimental Procedures”).

FIG. 4. Analysis of decay of poly(A+) β-globin/c-myc chimeric mRNA in S100. A portion of the β-globin/c-myc chimeric mRNA was treated with oligo(dT)_12–18, and RNase H to remove the poly(A) tract. This RNA was purified and incubated in cell-free mRNA decay reactions at 37°C for the indicated times with S100 from salt-washed polysomes. RNA from each time point was purified and subjected to deoxyoligonucleotide-directed RNase H mapping analysis for the c-myc 3′-UTR. RNA was fractionated in a denaturing 5% polyacrylamide gel and exposed to x-ray film. The arrows denote the 3′-UTR decay products.

DISCUSSION

A major focus toward understanding the control of mRNA turnover in mammalian cells has been the identification of proteins that bind to cis-acting instability elements (reviewed in Ref. 27). Here, I have described a novel cell-free system that utilizes soluble enzymatic activities to examine 3′ to 5′ decay using c-myc mRNA as a model. This system reconstitutes activities that promote removal of the poly(A) tract followed by degradation within the 3′-UTR. Additionally, these activities are 3′-UTR-dependent because the c-myc 3′-UTR directs 3′ to 5′ decay whereas β-globin mRNA sequences do not.

We and others have hypothesized that in cells the poly(A) tract of c-myc mRNA is protected by PAB and that early events in the decay of the mRNA may involve removal of PAB from the poly(A) tract so that it might be exposed to degrading enzymes (12, 24, 25). Removal of PAB may be promoted by interactions between trans-acting factors, such as AUFI in concert with other proteins, and the (A + U)-rich elements in the c-myc 3′-UTR. Although this may require association of c-myc mRNA with polysomes because partially purified preparations of AUFI can accelerate degradation of polysome-bound c-myc mRNA but not exogenous c-myc mRNA (28). However, it is important to note that the selective decay of c-myc mRNA in the system described here requires that PAB be effectively removed from the system with exogenous poly(A) competitor; without it, the c-myc 3′-UTR-containing mRNA is relatively stable (data not shown). Thus, the soluble-based decay system may be reconstituting enzymatic events that occur subsequent to the establishment of a ribonucleoprotein structure that permits access of the c-myc poly(A) tract to deadenylating nucleases(s) in the cell.

There are several reports in the recent literature of protein factors with properties that are consistent with their potential involvement in c-myc 3′ to 5′ mRNA decay processes. For example, Wahl and colleagues (29, 30) have purified and molecularly cloned a human, poly(A)-specific, 3′-exoribonuclease. Ectopic expression of the poly(A)-specific, 3′-exoribonuclease in nucleated oocytes from Xenopus rescues maturation-specific deadenylation. Caruccio and Ross (31) have purified a 3′-exoribonuclease believed to be a messenger RNase involved in histone mRNA decay; it also degrades poly(A). The deadenylase activity of both these enzymes is blocked at physiological salt concentrations by the binding of PAB to poly(A). However, decay of the body of most polyadenylated mRNAs is likely effected by factors distinct from poly(A)-specific ribonucleases. For example, whereas PAN activities mediate deadenylation of yeast mRNAs, 3′ to 5′ decay of the mRNA body is effected by a multi-component complex, known as the exosome, which contains multiple 3′- to 5′-exoribonucleases; the exosome is also found in mammalian cells (7, 32). Additional work will be required to determine whether any of these or perhaps distinct activities are responsible for 3′ to 5′ mRNA decay in mammalian cells.

Regardless of the identities of the 3′ to 5′ decay activities, there is likely some property of the c-myc 3′-UTR that permits action by the 3′ to 5′ degradation activities because β-globin mRNA is not rapidly deadenylated in the soluble-based system (Fig. 2B). It is interesting to note that deadenylation of the MFA2 mRNA in the yeast S. cerevisiae requires two (A + U)-rich regions of the 3′-UTR (33). Moreover, the PAB-dependent deadenylation of MFA2 mRNA in vitro by the purified poly(A) nuclease activity PAN appears to be a property conferred by the 3′-UTR in the absence of other purified protein components besides PAN (34). Clearly, resolution of this issue for 3′ to 5′ decay of c-myc mRNA will require reconstitution of mRNA decay from purified components to determine whether the enzyme activities recognize some feature(s) of the 3′-UTR or whether additional, sequence-specific RNA-binding proteins contribute to the specificity of these enzymatic processes.

In summary, I have utilized a soluble cell-free mRNA decay system to characterize enzymatic activities that are responsible for 3′ to 5′ decay of c-myc mRNA. These include a deadenylating activity and an activity that generates 3′ decay intermediates lying within the 3′-UTR. Future experiments will address the purification of these activities to reconstitute c-myc mRNA decay from purified components.

Acknowledgment—I thank Gerald Wilson for comments on the manuscript.

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