MOLECULAR AND CELLULAR BIOLOGY, July 1988, p. 2860–2868
0270-7306/88/072860-09$02.00/0

Vol. 8, No. 7

Differential Stability of c-*myc* mRNAS in a Cell-Free System

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Received 13 January 1988/Accepted 29 March 1988

We have developed a simple cell-free system for studying the stability of different mRNAs in vitro. We demonstrate that the threefold greater stability in vivo of truncated c-*myc* mRNA (lacking exon 1) compared with that of full-length c-*myc* mRNA is maintained in our in vitro system. Chimeric mRNAs in which the first exon of c-*myc* was fused to immunoglobulin Cα heavy chain or glyceraldehyde-3-phosphate dehydrogenase mRNAs were not rapidly degraded, demonstrating that c-*myc* exon 1 alone is not sufficient to tag mRNAs for rapid degradation. Competition experiments show that full-length c-*myc* mRNA is specifically recognized by a factor(s) responsible for its rapid degradation. This system will allow further characterization and purification of these factors.

It is becoming increasingly obvious that one important control point for regulation of some genes is the rate of mRNA degradation. Different mRNAs are known to have strikingly divergent half-lives in the same cells. For example, the mRNA for a metabolic enzyme, glyceraldehyde-3-phosphate dehydrogenase mRNA (GAPDH), has a half-life of many hours (30), whereas mRNA for the molecular protein encoded by the c-*myc* proto-oncogene has a half-life of only 10 to 30 min (8). Furthermore, it has been shown that the stability of some mRNAs changes in response to hormones (5, 17, 28), viral infection (20, 37), or cell growth conditions (18, 27).

The mechanisms which determine differential rates of mRNA degradation are poorly understood. *cis*-Acting elements such as specific sequences or particular secondary structures of the mRNA may influence the rate of mRNA decay. For example, an AU-rich sequence has been identified in the 3′ untranslated portion of the granulocyte-macrophage colony-stimulating factor mRNA that increases its rate of degradation as well as the rate of degradation of heterologous mRNAs to which it is fused (38). Similarly, a 106-base-pair (bp) sequence from the 5′ untranslated region and first exon of a β-tubulin gene was demonstrated to modulate β-tubulin mRNA stability (16). Furthermore, a putative loop structure is proposed to stabilize the 3′ portion of the polycistronic *rxcA* transcript from *Rhodopseudomonas capsulata* (2), and a repetitive extragenic palindromic sequence with a potential stem-loop structure has been shown to stabilize upstream mRNA segments in *Escherichia coli* transcripts (25). Specific *trans*-acting factors may also be important for differential mRNA stabilities. For example, immunoglobulin heavy- and light-chain mRNAs have the same structure throughout B-cell development, but there is increasing evidence that their mRNA half-lives increase markedly late in B-cell development (6). Also, it has been suggested that RNase III, which is specific for stem-loop structures, may catalyze the rate-limiting step of the decay of some mRNAs (1).

We have focused our attention on the stability of c-*myc* mRNA, since there is ample evidence that regulation of mRNA stability is an important control point for regulating the levels of c-*myc* gene product. When quiescent cells are stimulated with mitogens, c-*myc* mRNA levels increase in the case of CCL39 cells treated by thrombin and insulin (4), WEHI231 cells treated with anti-immunoglobulin (23), and rat skeletal muscle cells treated with serum factors or insulin (14). These have been shown to occur as a result of increased mRNA half-life. Similarly, when cells are induced to stop dividing and differentiate, c-*myc* mRNA levels fall. In the case of Daudi cells treated with interferon (9, 21) and F9 teratocarcinoma cells treated with retinoic acid (10), the decreases have been shown to involve increased c-*myc* RNA degradation.

Interestingly, further evidence of the importance of mRNA stability in c-*myc* regulation comes from B-cell tumors, where the c-*myc* gene is interrupted by chromosomal translocation. Due to translocation, the first untranslated exon of the normal c-*myc* mRNA is often replaced with other shorter 5′ untranslated sequences (7). It has been demonstrated by several groups that this truncated c-*myc* mRNA has a longer half-life than normal full-length c-*myc* mRNA (13, 32, 34), suggesting that part of the signal for c-*myc* mRNA degradation may reside in exon 1. The increased stability of truncated c-*myc* transcripts contributes to the increased levels of c-*myc* transcripts which are probably casual in the malignant transformation of these B-cell tumors. It is particularly significant that in COLO 320 cells, where both full-length and truncated forms of c-*myc* mRNA are present in the same cells, full-length c-*myc* mRNA is less stable than the truncated form (34).

To investigate possible *cis* elements and *trans*-acting factors which are important in regulating c-*myc* mRNA half-life we have developed an in vitro system for studying mRNA stability. In this report we describe the system and demonstrate that the differential stability observed between normal, full-length c-*myc* mRNA (F-*myc*) and truncated c-*myc* mRNA (T-*myc*) in vivo can be reproduced in vitro. c-*myc* mRNA degradation is not energy dependent in this system but is strongly dependent on the presence of divalent cations. Using chimeric mRNAs, we find that exon 1 alone is unable to tag heterologous mRNAs for rapid degradation, suggesting that the secondary structure of F-*myc* may be responsible for its rapid degradation. Finally, using F-*myc* and T-*myc* as competitors in the in vitro assay, we show that components of the crude cell extract which react preferentially with F-*myc* are responsible for its preferential degradation.

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MATERIALS AND METHODS

Construction of plasmids. To synthesize mRNA in vitro with T7 or Sp6 polymerase, DNA fragments were cloned into Bluescribe (vector cloning system) or Sp6-4 (24) vectors. The pBS-Fmyc plasmid, which contains DNA sequence corresponding to the full-length mouse c-*myc* mRNA cloned into the *Sma*I and *Xba*I sites of the Bluescribe vector adjacent to the T7 promotor, was used as template to transcribe F-*myc*. The 5′ end of the c-*myc* DNA (*Hae*III site to *Bam*HI site) was derived from a genomic c-*myc* clone (3) and starts 6 bp upstream of P1 (34); the 3′ end of the c-*myc* DNA fragment (*Bam*HI site to 3′ *Pst*I site) was derived from a c-*myc* cDNA clone, pmyc54 (39). The c-*myc* insert ends at the 3′ *Pst*I site and includes the poly(A) tract (see Fig. 1). The pBS-Tmyc plasmid, constructed by cloning the pmyc54 cDNA (*Sst*I site to the 3′-end *Pst*I site) into the *Sst*I site of the Bluescribe polylinker, was used as the template for T-*myc*. The 5′ end of the c-*myc* cDNA fragment starts at the *Sst*I site that is adjacent to the exon 2 of the c-*myc* gene, whereas the 3′ end is at the *Pst*I site (see Fig. 1). Plasmid Sp6GAPDH, which contains the whole 1.4-kilobase (kb) GAPDH cDNA fragment (15) (see Fig. 4a) cloned into the *Pst*I site of the Sp64 vector adjacent to the Sp6 promotor, was used as a template to transcribe GAPDH. pBSmyc1G, in which the c-*myc* *Pst*I fragment in pBS-Fmyc (see Fig. 4a) was replaced with the GAPDH *Pst*I fragment, was used as a template for the myc1G chimeric mRNA. pBSIg, which contains the 1.1- and 0.5-kb *Eco*RI fragments of the mouse immunoglobulin A heavy-chain gene cDNA (12) (see Fig. 4b) cloned into the *Eco*RI site of the Bluescribe vector, was used as a template for the immunoglobulin mRNA. pBSIgG, constructed by the fusion of the 0.8-kb 5′ potion of the immunoglobulin fragment (*Eco*RI site to *Pst*I site) with the whole GAPDH cDNA fragment (see Fig. 4a), was a template for the immunoglobulin G chimeric mRNA. pBSmyc1Ig, which contains 0.8-kb 5′ potion of c-*myc* DNA fragment (from the *Hae*III site 6 bp upstream of P1 to the first *Pst*I site) fused with 0.8-kb 3′ portion of the immunoglobulin DNA fragment, was the template for the myc1Ig chimeric mRNA (see Fig. 4b).

In vitro synthesis of α-^{32}P-labeled mRNAs. The plasmids were linearized by appropriate restriction at polylinker sites in the vectors 3′ of the cloned fragments. Transcription reactions were carried out for 1 h at 37°C in a mixture of 1 μg of the linearized DNA template, 40 mM Tris hydrochloride (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 0.4 mM ATP, CTP, and GTP, 0.04 mM UTP, 12 mM dithiothreitol, 10 U of RNasin, 10 μCi of [α-^{32}P]UTP, and 40 U of T7 polymerase. The reaction was stopped by the addition of 1 U of RNase-free DNase for 15 min at 37°C. The mRNA was phenol extracted and ethanol precipitated three times.

Similar transcription reactions were carried out with Sp6 polymerase. Linear DNA templates (100 μg/ml) were transcribed for 1 h at 37°C in a 10-μl mixture of 40 mM Tris hydrochloride (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 10 U of RNasin, 100 μg of bovine serum albumin per ml, 500 μM CTP, ATP, and GTP, 50 μM UTP, 10 μCi of [α^{32}P]UTP, and 3 U of Sp6 polymerase.

To synthesize 5′-capped mRNAs (26) the transcription reactions contained 1 μg of DNA template, 40 mM Tris hydrochloride (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 10 U of RNasin, 0.4 mM ATP, CTP, and m7G(5′)ppp(5′)G, 0.04 mM GTP and UTP, 10 μCi of [α-^{32}P]UTP, and 40 U of T7 polymerase.

Preparation of the cell extract. Mouse plasmacytoma P3XAg8.63 (P3X) cells (22) were cultured in Dulbecco modified Eagle medium with 10% horse serum. The cells were harvested at a concentration of 10^6 cells per ml while the cells were still in log phase growth. The cells were pelleted and washed twice with Dulbecco phosphate-buffered saline. The pellet was then suspended and incubated on ice for 5 min with two volumes of 0.25 M sucrose containing 2.5 μg of leupeptin per ml, aprotinin pepstatin, 150 μg of phenylmethylsulfonyl fluoride per ml, and 0.5 mM dithiothreitol. The lysate was centrifuged in a microfuge for 1 min. The supernatant was removed and further centrifuged in a microfuge for 30 min. The supernatant was quick-frozen on dry ice and stored at −70°C.

The lysosomal latency (percentage of the intact lysosomes) of the whole cell lysate before the second centrifugation was monitored by measuring the activity of β-hexosaminidase in the lysate with and without Triton X-100 (35). The assay mixtures contained 25 μl of the supernatant and 275 μl of 1.2 mM 4-methylumbeliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside in 13 mM citric acid–20 mM sodium phosphate (pH 4.4)–0.25 M sucrose. After 10 min at 37°C, the reaction was quenched with 1 ml of glycine-carbonate buffer (pH 10). The concentration of the liberated 4-methylumbelliferone was measured in a Farrand MKI spectrofluorometer (365-nm excitation and 450-nm emission). The percentage of lysosomes that were broken was determined from the ratio of the free to total β-hexosamimdase activity.

Isolation of polysomes. P3X cells, harvested as described above, were suspended in buffer A (1 mM potassium acetate, 1.5 mM magnesium acetate, 2 mM dithiothreitol, and 10 mM Tris hydrochloride [pH 7.6]) at approximately 10^8 cells per ml; cells were then broken by 30 vigorous strokes with a tight-fitting Teflon pestle homogenizer. The lysate was centrifuged in a Sorvall SS34 rotor at 10,000 rpm for 10 min. The supernatant was removed to a SW60 Ultraclear tube (Beckman Instruments, Inc.) and layered over a cushion of 1.5 ml of 30% sucrose equilibrated with buffer A. The tubes were centrifuged in a SW60 rotor for 3 h at 36,000 rpm. The pellet was rinsed twice with buffer A. The polysomes were suspended in a small volume of buffer A to a concentration of 150 A_{260} units per ml (1-cm light path). The recovery was approximately 3 A_{260} units of polysomes per 10^8 cells. The polysomes were aliquoted and stored at −70°C.

In vitro mRNA degradation assay. mRNA (10 pg) synthesized in vitro (calculated from the specific activity of ^{32}P) was incubated at 37°C with 50 μg of P3X extract per ml in 50 μl of degradation reaction buffer (20 mM Tris hydrochloride [pH 8.0], 5 mM KCl, 0.15 mM NaCl). Small samples of the reaction mixture were taken out at different times and quenched by adding 100 μl of stopping solution (2 mM EDTA, 20 mM Tris hydrochloride [pH 8], 0.5% sodium dodecyl sulfate, 50 μl phenol, and 50 μl of chloroform-isoamyl alcohol [24:1, vol/vol]). The phenol-extracted reaction samples from each time point were precipitated with ethanol and resolved by electrophoresis through an agarose-formaldehyde gel (1% agarose, 6.2% formaldehyde, 20 mM morpholinepropanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA). Radioactivities were visualized by autoradiography on X-ray film (Eastman Kodak Co.) and quantified by silver-grain elution (40). Bands corresponding to intact mRNAs from the developed X-ray film were excised and eluted with 1 M NaOH overnight at 25°C. Similar sections were excised from each lane. The absorbance was then measured at 500 nm.

The mRNA degradation in the polysome fraction was carried out like the degradation in the P3X extract, except

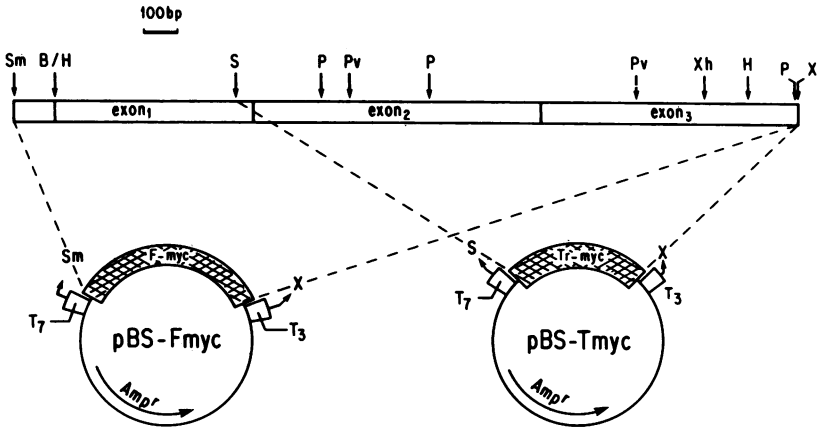

FIG. 1. Structure of pBS-Fmyc and pBS-Tmyc plasmids. Mouse c-*myc* DNA fragments were cloned into Bluescribe vectors. pBS-Fmyc contains a c-*myc* DNA fragment in which the 5′ end is 6 bp 5′ of P1 and the 3′ end is at the *Pst*I site at the end of the cDNA clone pmyc54 (39). The construct contains all of the c-*myc* exons and the poly(A) tail. pBS-Tmyc contains a c-*myc* DNA fragment in which the 5′ end starts at the *Sst*I site that is adjacent to the second exon of the c-*myc* gene and the 3′ end is the same that in pBS-Fmyc. In both plasmids the T7 promoters are adjacent to the c-*myc* DNA fragment. The in vitro transcription templates were made by linearizing the plasmids at the end of the c-*myc* DNA fragments. Abbreviations: B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; S, *Sst*I; Sm, *Sma*I; X, *Xba*I; Xh, *Xho*I.

that the P3X extract was replaced with polysomes (0.6 A_{260} units per ml).

RESULTS

Differential c-*myc* mRNA stabilities are observed in the in vitro system. Crude cell extracts were prepared from plasmacytoma P3X cells which were gently lysed by treatment on ice with hypotonic sucrose in the presence of protease inhibitors. Nuclei, mitochondria, and lysosomes were removed by centrifugation, and the resulting supernatant was incubated with labeled mRNA.

To ascertain whether our in vitro system was able to reproduce the well-documented difference between c-*myc* mRNA stabilities observed in vivo, we studied the stability of T-*myc* and F-*myc* compared with a control mRNA, GAPDH. Labeled mRNAs synthesized in vitro with cloned genes and bacteriophage polymerases were incubated with crude cell extract, and their degradations over time were assayed by electrophoresis and autoradiography. Figure 1 shows the plasmid constructs that were used for in vitro synthesis of labeled F-*myc* and T-*myc*. F-*myc* contains sequences transcribed from the full-length c-*myc* cDNA [exons 1, 2, and 3 and the poly(A) tract] and is exactly similar in sequence to in vivo full-length c-*myc* mRNA. T-*myc* contains sequences from exons 2 and 3 and the poly(A) tract. Therefore, T-*myc* is similar to truncated forms of c-*myc* mRNAs which initiate within the first intron or at the beginning of the second exon of c-*myc* (7). In most experiments, neither F-*myc* nor T-*myc* contained a 5′ cap.

Figure 2A shows the decay of these two mRNAs and of GAPDH mRNA after incubation with a crude cell extract from murine plasmacytoma P3X cells. Quantitation of the amount of radioactivity remaining in intact mRNA, by measurement silver grains (40), allowed calculation of the mRNA half-lives (Fig. 2B). The half-lives of F-*myc*, T-*myc*, and control GAPDH mRNAs in vitro were 2.5 to 15, 11 to 40, and 16 to 63 min, respectively. The mRNA half-life ratio of T-*myc* to F-*myc* was 3.3 ± 0.8 (n = 7); that of GAPDH to F-*myc* was 4.2 ± 0.7 (n = 3). These data show that although the absolute half-life values for F-*myc* and T-*myc* are somewhat variable and usually shorter than those observed in vivo, their relative values are quite similar, i.e., T-*myc* is about three times more stable than F-*myc*, and both are degraded more rapidly than GAPDH. Thus we conclude that our in vitro system is capable of reproducing the differential stability of T-*myc* and F-*myc* observed in vivo and should allow us to study this phenomenon in detail.

Biochemical requirements of the in vitro system. We wished to study various requirements and characteristics of the in vitro RNA degradation system. To understand the level of nonspecific lysosomal RNase in our system, we performed a lysosome latency assay (35). RNase released from broken lysosomes is probably the primary source of nonspecific

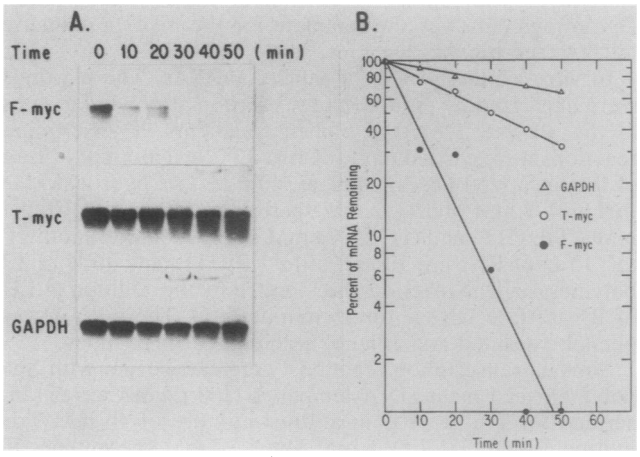

FIG. 2. mRNA stability in the in vitro system. mRNA (10 pg) was incubated with P3X extract (50 μg/ml) at 37°C in a 50-μl reaction mixture (20 mM Tris hydrochloride (pH 8.0), 5 mM KCl, 0.15 mM NaCl). (A) At different time points samples were analyzed by electrophoresis through an agarose-formaldehyde gel and visualized by autoradiography. (B) Determination of mRNA half-lives. The blackened bands which represent the intact mRNAs from the film were excised and eluted by 1 M NaOH. A_{500} was measured. The percentage of the remaining mRNA, calculated by the ratio of the absorbance of 500 nm at a given time point relative to that at time zero was plotted.

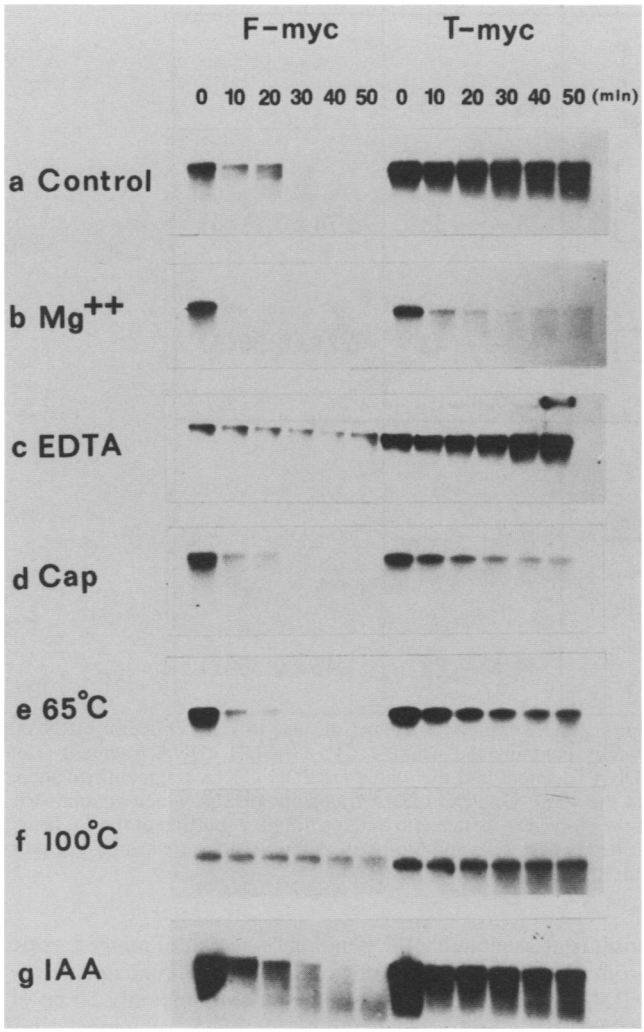

FIG. 3. mRNA stabilities under varied conditions in the in vitro system. mRNA (10 pg) synthesized in vitro was incubated with P3X cell extract (50 μg/ml) in a 50-μl reaction mixture containing 20 mM Tris hydrochloride (pH 8.0)–5 mM KCl–0.15 mM NaCl (a, c, e, f, g), 20 mM Tris hydrochloride (pH 8.0)–5 mM $MgCl_2$–5 mM KCl–0.15 mM NaCl (b), or 20 mM Tris hydrochloride (pH 8.0)–5 mM KCl–20 mM EDTA–0.15 mM NaCl (c). Capped mRNAs were used (d) in contrast to the usage of the uncapped mRNAs (a, b, c, e, f, g). The P3X extract was incubated at 65°C for 10 min (e) or at 100°C for 15 min (f) or treated twice with 0.03 g of iodoacetic acid (pH 5.0) per ml at 55°C for 40 min (g) before the degradation reaction.

RNase in the in vitro system (Rome, personal communication). The percentage of intact lysosomes was determined for our cell extracts before the high-speed centrifugation step that pellets intact lysosomes. This assay showed that when cells were lysed in 0.25 M sucrose, 85 to 90% of the lysosomes in our extracts before high-speed centrifugation remained unbroken (data not shown).

The effect of 7-methyl G capping on the differential stability of F-*myc* and T-*myc* was investigated. We used the conditions described by Nielsen and Shapiro (26), which give approximately 80% capping of mRNAs synthesized in vitro, and then carried out the degradation assay. Results of a typical experiment are shown in Fig. 3d, and the results of three experiments are presented in Table 1. These results

TABLE 1. mRNA half-lives of capped F-*myc* and capped T-*myc* in vitro[a]

Expt	Half-life (min)		Half-life ratio (T-*myc*/F-*myc*)
	F-*myc*	T-*myc*	
1	4.3	8.6	1.9
2	5	25	5
3	4.5	15.8	3.5

[a] Capped mRNAs were prepared and incubated with crude P3X cell extracts; the half-lives and half-life ratios were then determined as described in Materials and Methods.

show that neither the absolute half-lives nor the half-life ratio of T-*myc* to F-*myc* (3.5 ± 1.3) was significantly different with capped mRNAs compared with that with uncapped mRNAs. Since our primary interest is in the differential stability of F-*myc* and T-*myc*, we did not use capped mRNAs for the remaining experiments.

Our standard assay conditions did not contain added Mg^{2+} but did contain Mg^{2+} that was present in the cells before lysis (estimated to be 0.5 to 1 mM Mg^{2+} in the final extract). When the degradation assay was carried out in the presence of 20 mM EDTA, both F-*myc* and T-*myc* became more stable (Fig. 3c). Alternatively, when 5 mM Mg^{2+} was added to the extracts (Fig. 3b), both mRNAs were degraded so rapidly that it was not possible to distinguish any difference in their rates of degradation. Thus it is evident that degradation of mRNA in this crude system is dependent on a divalent cation concentration. Since degradation in the presence of added Mg^{2+} was extremely rapid, we could not determine whether it was specific, nonspecific, or both.

The degradative ability of the extracts could be inactivated by heating (Fig. 3e and f). Although heating for 10 min at 65°C did not diminish the rate of mRNA degradation, boiling for 15 min essentially inactivated all degradative activity. Although nonspecific occlusion of a heat-stable protein in denatured protein precipitates after boiling cannot be formally ruled out, we think it is unlikely because our protein mixture was very dilute (0.05 mg/ml) and no precipitate was observed after boiling. Thus we conclude that one or more key component of the degradative process is heat sensitive and thus distinct from RNase A.

Treatment with iodoacetic acid at pH 5 is known to inactivate certain RNases such as pancreatic RNase while most other proteins remain active. This mild iodoacetic acid treatment of the extracts did not decrease the preferential stability of T-*myc* relative to F-*myc*; the average half-life ratio of T-*myc* to F-*myc* was approximately 6 in two experiments (representative experiments shown in Fig. 3g). It is not clear whether the apparent twofold stabilization of T-*myc* after iodoacetic acid treatment is significant. More extensive treatment of the extract with iodoacetic acid inactivated all degradative activity (data not shown). These results are also consistent with the notion that RNase A-type enzymes are not important for determining the differential stability of F-*myc* and T-*myc*.

One would expect that length should be one factor in determining degradation rate, since longer mRNAs provide a larger target for nonspecific endoribonucleases. Indeed many short mRNAs are more stable in our system than longer ones. However, the small difference in length between T-*myc* and F-*myc* (1.7 and 2.2 kb, respectively) cannot account for a threefold difference in stability. Furthermore, a chimeric mRNA having *myc* exon 1 fused to GAPDH had a longer half-life (15 to 38 min) than either

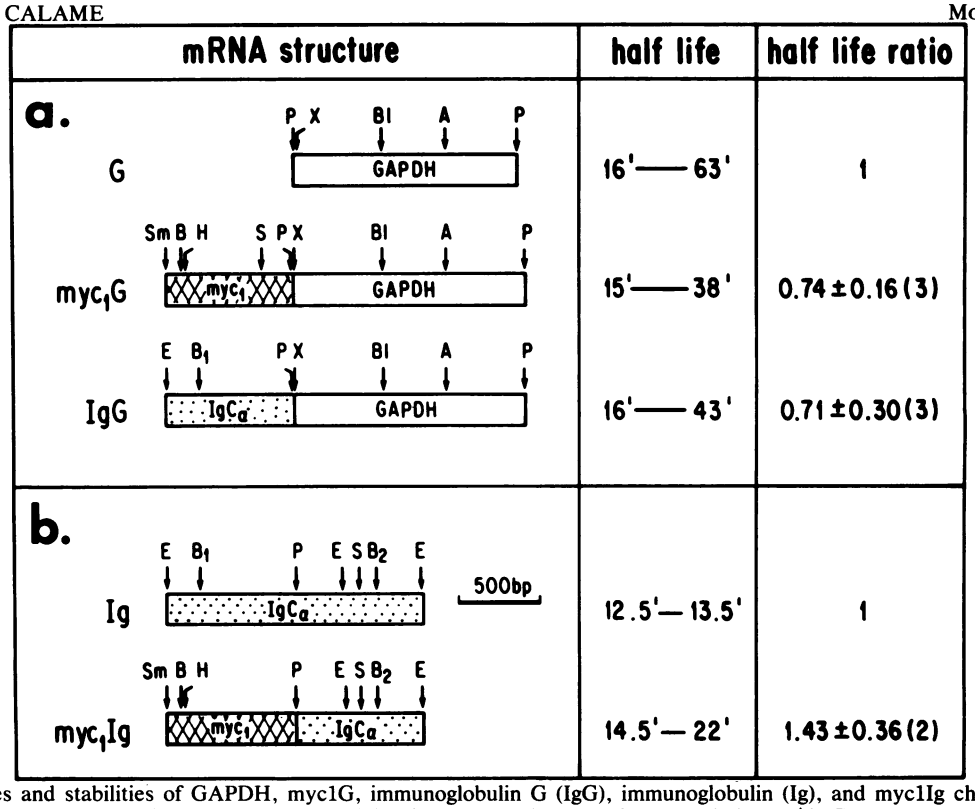

FIG. 4. Structures and stabilities of GAPDH, mycIG, immunoglobulin G (IgG), immunoglobulin (Ig), and mycIIg chimeric mRNAs. mRNAs were transcribed in vitro from the templates of Sp6GAPDH, which contains the whole 1.4-kb GAPDH cDNA fragment (15); pBSmycIGAPDH, which contains 0.8 kb of the 5′ portion of the c-*myc* DNA fragment and the whole GAPDH cDNA fragment; pBSIgG, which contains 0.8 kb of the 5′ portion of the Cα cDNA fragment (12) and the whole GAPDH cDNA fragment; pBSIg, which contains the Cα gene fragment; and pBSmycIIg, which contains the 0.8-kb 5′ portion of the c-*myc* DNA fragment and the 0.8-kb 3′ portion of the Cα gene. The half-lives and half-life ratios of these mRNAs were determined as described in Materials and Methods. Abbreviations are as in Fig. 1 plus the following: A, *Apa*I; B_1, *Bst*eI; B_2, *Bst*eII; Bl, *Bal*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sst*I; Sm, *Sma*I.

F-*myc* or T-*myc* mRNA, although its length is 2.2 kb, the same as F-*myc* (Fig. 4). In addition, we found several other cases where shorter mRNAs were degraded more rapidly than longer ones (data not shown). Thus we conclude that other effects, possibly recognition of specific sequences or structures by exoribonucleases or other proteins, can have larger effects than does length on degradation rates.

Finally, we prepared a crude polysome fraction by the method of Ross et al. (36) and compared the stabilities of T-*myc*, F-*myc*, and GAPDH mRNA upon incubation with this fraction (Table 2). The average relative half-life of T-*myc* to F-*myc* was 2.5; both were less stable than GAPDH. Thus, the in vitro stabilities of these three mRNAs are similar in the polysome fraction to their stabilities in the crude cell extract (see above).

c-*myc* exon 1 cannot tag heterologous mRNAs for rapid degradation. Truncated c-*myc* transcripts in vivo lack the first c-*myc* exon and usually contain a variable but shorter 5′ untranslated sequence, depending on which of many cryptic promoters initiates their transcription (33). Truncated c-*myc* mRNA synthesized in vitro (T-*myc*) contains only 50 bp of the first exon and contains no additional 5′ sequences; this form of mRNA is also more stable than F-*myc*. Thus, it seemed reasonable to suspect that exon 1 alone might provide a tag to c-*myc* mRNA which targeted it for rapid degradation. To test this hypothesis, we wished to attach exon 1 to heterologous mRNAs and determine whether exon 1 was sufficient to target them for rapid degradation. Accordingly, several plasmids were constructed so that chimeric mRNAs could be synthesized in vitro (Fig. 4a). c-*myc* exon 1 and a portion of exon 2 (814-bp *Pst*I fragment; Fig. 1) was attached to the GAPDH mRNA and to the 3′ portion of immunoglobulin heavy-chain alpha mRNA; as a control, we attached the 5′ portion of immunoglobulin alpha-chain mRNA to the GADPH mRNA. Figure 4b shows the compilation of several degradation experiments with each chimeric mRNA in vitro. The results show that the addition of c-*myc* exon 1 does not significantly increase the rate of degradation of these chimeric mRNAs. Thus we conclude that exon 1, although necessary, is not sufficient to tag heterologous mRNAs for rapid degradation in vitro.

TABLE 2. mRNA half-lives after incubation with a crude polysome fraction[a]

Expt	Half-life (min)			Half-life ratio	
	F-*myc*	T-*myc*	GAPDH	T-*myc*/ F-*myc*	GAPDH/ F-*myc*
1	12	21	>60	1.75	>6
2	5.5	17.5	ND	3.2	ND
3	6.5	17.0	ND	2.6	ND

[a] A polysome fraction similar to that of Ross et al. was prepared as described in Materials and Methods. ND, Not determined.

Competition experiments demonstrate that factors in the crude cell extract which specifically recognize F-*myc* are responsible for its rapid degradation. We reasoned that if one or more specific factors were responsible for the differential degradation of F-*myc* relative to T-*myc*, the factor(s) should recognize one or the other of these mRNAs specifically, either to degrade F-*myc* or to protect T-*myc*. We also

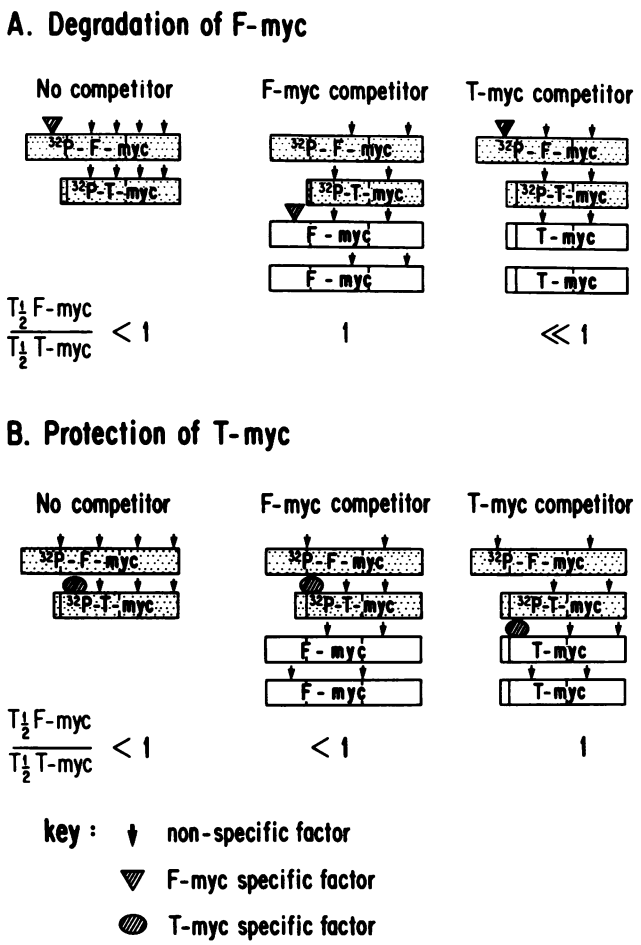

FIG. 5. Two models for the differential stability of the F-*myc* and T-*myc* in the in vitro system. Both specific and nonspecific factors are assumed to exist in the system. (A) A specific factor is presumed to recognize F-*myc* and target its rapid degradation. The factor competes with F-*myc*, decreasing the differential stability of F-*myc* and T-*myc*. (B) A specific factor is presumed to protect T-*myc*. Although F-*myc* cannot compete successfully with the protecting factor, T-*myc* can, decreasing the differential stability of F-*myc* and T-*myc*.

realized that it was likely that nonspecific RNA degradation occurred in our system due to 10 to 15% lysosomal breakage and the probable presence of nonspecific RNases. We hoped to distinguish between general and specific degradation and to determine the target of specific factors by carrying out competition studies with a variety of unlabeled competitor RNAs.

The rationale for these experiments is illustrated in Fig. 5, where two possible models are considered. Both specific and nonspecific factors are presumed to be present in the system (nonspecific RNases are indicated by small arrowheads). First (Fig. 5A), if a specific degradative factor(s) recognized F-*myc* (indicated by large arrowhead), it would compete with excess unlabeled F-*myc*, and the differential stability of F-*myc* and T-*myc* would be lost. In this case T-*myc* or any nonspecific mRNA would compete with nonspecific factors and result in overall decreased degradation but an increased degradation of F-*myc* relative to T-*myc*. Alternatively (Fig. 5B), if a specific protecting factor recognizes T-*myc* (indicated by large circle), it would compete with T-*myc*, and the differential stability between T-*myc* and F-*myc* would be lost. F-*myc* or another nonspecific RNA would compete with nonspecific factors and slow degradation but not alter the differential stability of T-*myc* and F-*myc* (Fig. 5B). The only assumption made as to the nature or activity of putative factors in these models is their ability to recognize and target, for degradation or protection, respectively, F-*myc* or T-*myc*. In all cases competitor mRNAs would be expected to stabilize nonspecific degradation of both F-*myc* and T-*myc*.

TABLE 3. mRNA half-lives in the competition experiment[a]

Expt	Competitor	Half-life (min)		Half-life ratio (T-*myc*/F-*myc*)
		F-*myc*	T-*myc*	
1	None	10	18	1.8
	F-*myc*	45	35	0.78
	T-*myc*	ND	ND	ND
	Poly(A)$^+$ RNA	16.5	75	4.5
2	None	14	29	2.1
	F-*myc*	41	43	1.0
	T-*myc*	19	55	2.9
	Poly(A)$^+$ RNA	20	100	5
3	None	5.9	15.0	2.9
	F-*myc*	20	17	0.85
	T-*myc*	16	76	4.8
	Poly(A)$^+$ RNA	6	42	7

[a] α-^{32}P-labeled mRNA (10 pg) was incubated with P3Xcell extract with and without 100 pg of unlabeled competitor RNA. Half-lives and half-life ratios were determined as described in Materials and Methods. ND, Not determined.

The results of competition experiments are shown in Table 3. Unlabeled competitor T-*myc* and F-*myc* mRNAs were synthesized in vitro under standard conditions. As an additional control for nonspecific RNA we also used total poly(A)$^+$ mRNA isolated from P3X cells as a competitor. The results show that competition with F-*myc* abolished the difference in stability between F-*myc* and T-*myc*, whereas competition with T-*myc* or poly(A)$^+$ mRNA increased the stability of T-*myc* relative to F-*myc*. All three competitors stabilized both mRNAs. This is the result predicted by model A in Fig. 5. The crude extracts themselves contain poly(A)$^+$ RNA, but most of it is probably associated with ribosomes or other proteins and does not compete as efficiently as RNA made in vitro, which is not associated with protein. Thus we conclude that both general and specific degradation are occurring in our system. The factor(s) responsible for the specific differential stability clearly recognizes F-*myc*, since differential stability disappears only when F-*myc* is used as a competitor. These data do not distinguish between the alternative possibilities that the factor(s) is (i) an RNase or (ii) another molecule which targets RNases to F-*myc*.

DISCUSSION

Characteristics of the in vitro mRNA degradation system. We describe a very simple cell-free system in which the differential stability of F-*myc* versus T-*myc* observed in vivo is maintained in vitro. The absolute half-lives of the various mRNAs tested in this system are lower than those reported in vivo. This is not surprising, because the ratio of cell protein to labeled mRNA is much higher in vitro than in the cell. In addition, the labeled mRNA is not associated in vitro with ribosomes or other proteins which might protect against

degradation. Finally, there is 10 to 15% lysosomal breakage during our extract preparation, which may expose the test mRNAs is nonspecific lysosomal RNases that are not present in vivo, although lysosomal enzymes would not have high activity in the reaction buffer at pH 8. However, the fact that the relative stabilities of F-*myc* and T-*myc* are retained in this system supports the view that most of the factors which are responsible for their differential stability in vivo are retained in our crude cell extract.

Ross et al. have previously described a system in which mRNAs synthesized in vitro are incubated in a cell-free polysome fraction (36). We have prepared a polysome fraction by their procedure and tested the relative stability of F-*myc* and T-*myc*. Using this polysome fraction in place of our crude cell extract, we obtained half-life ratios for F-*myc* and T-*myc* mRNAs that were similar to that obtained with our standard crude extract, which also contains ribosomes and polysomes (Table 2). Thus, mRNA degradation in the polysome fraction may involve factors similar or identical to those used in our crude extract. Our results suggest, however, that association of mRNA with ribosomes is not necessary to reproduce the differential degradation rates observed in vivo, although translation factors or dissociated ribosomal proteins may be important in our extracts. It will be necessary to characterize and purify components of both systems further to understand their exact relationship.

Since there is evidence that 5′ capping does confer stability to mRNAs (11, 29), we were surprised that capping appeared to have little effect on the stability of normal and truncated *myc* RNAs in our in vitro system. The explanation for this result is not clear, but lack of capping effect suggests that the in vitro system does not completely reproduce the in vivo situation. However, the absence of a capping effect does not affect the differential degradation of full-length versus truncated c-*myc* mRNA in vitro, which we have shown reproduces that observed in vivo. This is consistent with the fact that both mRNAs are capped in vivo and the expectation that the mechanisms responsible for their differential degradation must recognize some feature of their primary or higher-order structure which is not in common between the two. Thus, even though it may not completely reproduce in vivo conditions, the in vitro system will be useful for studying factors which are important for differential degradation of *myc* mRNAs.

The sensitivity of mRNA degradation in our system to diavalent cations is striking. Although phosphotransferase RNases, which act through a 2′-3′ cyclic intermediate, do not require divalent cations, phosphodiesterase RNases do require Mg^{2+} or Ca^{2+} (1). In addition, Mg^{2+} is known to modify the structure of RNA, and this effect on the substrate can cause enhancement or inhibition of RNAse activity as well (1). The data do not distinguish between effects on RNA structure and RNase activity, and it is reasonable to expect that both may be important. The data are consistent with a role for mRNA structure and with the involvement of phosphodiesterase-type RNases in the degradation process.

Heating the crude extract at 100°C for 15 min strongly increased the stability of all mRNAs, indicating that heat-sensitive proteins are probably involved. It is known that RNases such as pancreatic RNase are stable to boiling; other RNases, both cyclizing and phosphodiesterase types, are heat labile (1). Furthermore, RNasin, an inhibitor of pancreatic-type RNase, has little influence on the degrative properties of our system (data not shown). Thus, the ability to inactivate degradative activity by heating implies that pancreatic-type RNases are not the key degragative component in our system. However, it does not allow us to distinguish other types of RNases or between RNases and other types of proteins which might be important for mRNA degradation.

Sensitivity to mild iodoacetic acid, however, is characteristic of cyclizing phosphotransferase RNases. Since mild iodoacetic acid treatment of cell extracts does not decrease the differential stability between F-*myc* and T-*myc* but appears to stabilize T-*myc* relative to F-*myc*, we conclude that the preferential degradation of F-*myc* is not mediated by a cyclizing phosphotransferase RNase and may be mediated by a noncyclizing phosphodiesterase (most exonucleases are of the latter type). However, due to the crude preparation of the cell extracts, we have yet not determined which kind of RNase is responsible for the perferential F-*myc* degradation.

Roles of *cis*- and *trans*-acting factors in c-*myc* mRNA degradation. We were unable to increase the degradation rate of immunoglobulin heavy-chain or GADPH mRNAs by the addition of the first exon of c-*myc* mRNA. Similar results have been reported recently for in vivo studies where the stability of different chimeric mRNAs containing exon 1 was studied (19, 33). This agreement between our in vitro results and observations of mRNA half-lives in vivo strengthens the assumption that similar factors are responsible for mRNA degradation in the two systems. The inability of exon 1 to tag heterologous mRNAs for rapid degradation rules out the simple model which hypothesizes that a sequence within this exon is recognized by a specific RNase. It suggests that a more complex mRNA structure, which depends on exon 1 but includes other c-*myc* sequences as well, must determine the rapid degradation of F-*myc*. This notion is strengthened by our observation that c-*myc* mRNAs with the same 5′ end and different 3′ ends transcribed from pBs-Fmyc templates digested with various restriction enzymes before in vitro transcription showed higher stability than that of F-*myc* (data not shown). The model is also consistent with the importance of mRNA secondary structure suggested by the divalent cation dependence we have observed.

It is interesting to note that both F-*myc* and T-*myc* contain an AU-rich sequence at their common 3′ end which is similar to the sequence that has been shown to tag granulocyte-macrophage colony-stimulating factor mRNA for rapid degradation (38). Also, sequences that are primarily responsible for the short half-life of c-*myc* mRNA were localized recently to a region of 140 bp in the 3′ untranslated region (19). Thus, the AU-rich sequence is likely to be involved in the degradation of both F-*myc* and T-*myc*, but it is unlikely that the sequence itself could be responsible for their differential degradation rates since it is present in both mRNAs.

Our competition experiments clearly demonstrate that the activity responsible for the differential degradation of F-*myc* versus T-*myc* in vitro recognizes F-*myc*. Presumably this F-*myc*-specific factor is involved in the regulation of F-*myc* levels in cells representing different developmental or growth states. It will be interesting to use our in vitro assay to compare the half-life of F-*myc* incubated with extracts from different cells or cells before and after treatment with mitogens or inducers of differentiation.

What is the nature of the factor(s) which specifically recognizes F-*myc* and targets its degradation? The iodoacetic acid results suggest that it is not a cyclizing RNase, although this type of activity may be important for less specific degradation. Our results do not allow us to distinguish between a noncyclizing RNase and some other protein which might recognize F-*myc* and target its degradation. If an RNase is involved, it will be interesting to determine whether it is a 3′ or 5′ exoribonuclease or an endoribonu-

clease. These questions await fractionation and further characterization of our crude extracts.

In summary, our results suggest that degradation of c-*myc* mRNA is a complex process which involves both general and specific factors. We have demonstrated that the differential stability of F-*myc* versus T-*myc* observed in vivo is maintained in our system and that factor(s) responsible for this differential stability specifically recognize F-*myc*. The system which we have developed should allow the factor(s) responsible for both general and specific mRNA degradation to be purified and characterized in more detail.

ACKNOWLEDGMENTS

We thank P. Fort and K. Marcu for plasmid pRGAPHD 13, and we appreciate the help of L. Rome with the lysosome latency assays. We are grateful to our colleagues Dohn Glitz, Leonard Rome, Emil Kakkis, Larry Feldman, Arnold Berk, and Edward De Robertis for helpful discussion and critical reading of this manuscript and Betty Tsao, Craig Petersen, Suzanne Eaton, Skye McDougall, Mike Farrell, Karen Riggs, and Christopher Roman for helpful discussion.

This work was supported by Public Health Service grants CA38571 and GM29361 from the National Institutes of Health. K.C. is a Leukemia Society Scholar.

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