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The Half-Life of c-*myc* mRNA in Growing and Serum-Stimulated Cells: Influence of the Coding and 3′ Untranslated Regions and Role of Ribosome Translocation

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c-*myc* mRNA contains at least two discrete sequence elements that account for its short half-life, one in the 3′ untranslated region and the other in the carboxy-terminal coding region (coding-region determinant). To investigate the function of each determinant, one or both were fused in frame to portions of a gene encoding long-lived β-globin mRNA. Each chimeric gene was stably transfected into HeLa and NIH 3T3 cells and was transcribed from a constitutive cytomegalovirus promoter or from a serum-regulated c-*fos* promoter, respectively. The steady-state levels of the chimeric mRNAs in exponentially growing HeLa cells were compared, and their half-lives were measured by two independent methods: (i) in actinomycin D-treated HeLa cells and (ii) after serum addition to starved 3T3 cells. By each method, mRNAs containing either instability determinant were less stable than β-globin mRNA. mRNA containing only the c-*myc* 3′ untranslated region was not significantly more stable than mRNA with both determinants. In a cell-free mRNA decay system containing polysomes from transfected HeLa cells, mRNA containing the coding-region determinant was destabilized by addition of a specific RNA competitor, whereas mRNA containing only the 3′ untranslated region was unaffected. When a stop codon was inserted upstream of the coding-region determinant, the chimeric mRNA was stabilized approximately twofold. These and other data suggest that degradation involving the coding-region determinant occurs most efficiently when ribosomes are translating the determinant.

The c-*myc* proto-oncogene encodes phosphoproteins that influence cell proliferation, differentiation, and neoplastic transformation (reviewed in references 9, 10, 27, and 34). During cell growth and differentiation, c-*myc* gene expression is regulated both transcriptionally and posttranscriptionally. Depending on cell growth conditions, the mRNA half-life can vary from approximately 30 to 120 min. To map c-*myc* mRNA instability determinants, investigators have expressed modified c-*myc* genes in transfected cells and measured the half-lives of the modified mRNAs by various techniques. In some cases, mRNA lacking a particular region was analyzed; in other cases, a chimeric mRNA, in which a segment from c-*myc* mRNA was inserted within a stable mRNA, was expressed. Both approaches revealed at least two instability determinants, one in the 3′ untranslated region (3′-UTR) and the other in the carboxy-terminal portion of the coding region (the coding-region determinant [CRD]). Deleting either segment prolonged the mRNA half-life but only by a factor of 5 or less. The CRD was first detected indirectly through the analysis of 3′-UTR deletions. c-*myc* mRNA lacking most of its 3′-UTR was more stable than wild-type c-*myc* mRNA but was still significantly less stable than most other mRNAs. Therefore, the 3′-truncated mRNA retained an instability determinant (1, 4, 17, 22, 24, 27a, 39). Subsequent experiments demonstrated directly that the carboxy-terminal coding region of the mRNA contains an instability determinant (40).

These observations raised three questions. Why does c-*myc* mRNA contain two instability-determining segments? Which *trans*-acting regulatory factors interact with each segment, and how and under what circumstances do they control mRNA stability? Does each instability determinant specify a unique degradation pathway? Experiments with in vitro mRNA decay extracts have suggested that each instability-determining segment can function independently, is recognized by different regulatory/degradation factors, and specifies a unique decay pathway.

Two putative regulatory factors, one cytosolic and the other mRNA or ribosome associated, have been analyzed by using a cell-free mRNA decay system. When the decay reaction mixtures contained only polysomes, placental RNase inhibitor, buffer, and an ATP-generating system, polysome-associated c-*myc* mRNA was degraded with a half-life of approximately 40 min (6). When the reaction mixtures were supplemented with a crude postpolysomal supernatant fraction (cytosol or S130) from exponentially growing cells, the c-*myc* mRNA half-life was reduced to 5 min or less (7). These and other experiments suggested that the cytosol contains a labile destabilizing factor. A separate protein factor was identified when the decay reaction mixtures were supplemented with an exogenous 182-nucleotide (nt) sense strand competitor RNA corresponding to the carboxy-terminal segment of the mRNA coding region. Polysome-bound c-*myc* mRNA was destabilized eightfold by this CRD RNA competitor (3). RNA competitors from other regions of c-*myc* mRNA or from different sources (globin or a plasmid vector) did not affect c-*myc* mRNA stability. A polysome-associated chimeric mRNA, containing the c-*myc* CRD inserted in frame into the β-globin coding region, was also destabilized by the c-*myc* competitor RNA. Gel shift and UV cross-linking experiments revealed an ~70-kDa polysomal protein that binds to the c-*myc* mRNA CRD.

To account for these observations, we proposed that the

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CRD-binding protein (CRD-BP) normally binds to the c-*myc* CRD and protects it from cleavage. We also proposed that the CRD competitor RNA was titrating the CRD-BP from the mRNA. Consistent with this interpretation, endonucleolytic cleavage products were detected in reaction mixtures containing the CRD competitor RNA, presumably because, when the CRD-BP was titrated from the mRNA, a cleavage site within the CRD became exposed to a ribosome-bound endonuclease.

To correlate the transfection and in vitro results summarized above and to define more precisely how each determinant functions, we constructed chimeric genes in which one or the other determinant was inserted within the human β-globin gene. Each gene was placed downstream of either a cytomegalovirus (CMV) immediate-early promoter or the c-*fos* promoter and was transfected into HeLa or NIH 3T3 cells, respectively. Drug-resistant pools were selected, and mRNA decay rates were measured in cells and in vitro. The effect of placing a nonsense mutation upstream of the CRD was also investigated. The data indicate that each determinant can function on its own, that the 3′-UTR determinant is the stronger destabilizing element, at least under the conditions tested, and that the CRD must be translated to function most efficiently.

MATERIALS AND METHODS

Cell culture and transfections. HeLa cells were maintained in exponential growth in alpha modified Eagle's medium containing 10% calf serum (GIBCO), penicillin, and streptomycin. Mouse NIH 3T3 cells were maintained in exponential growth in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum, penicillin, and streptomycin. Actinomycin D was dissolved in H_2O and added to cells at 5 μg/ml.

HeLa cells (5×10^6 per 100-mm dish) were lipofected with a mixture of 50 μg of Lipofectin reagent (GIBCO BRL) plus 20 μg of plasmid DNA as specified by the manufacturer. All plasmids contained a hygromycin phosphotransferase gene for selection (15). Cells were cultured for 48 h without selection and then in growth medium supplemented with hygromycin B (400 μg/ml; Boehringer Mannheim). After 9 to 12 days, the cells were harvested, layered over a 1-ml cushion of Ficoll-Paque (Pharmacia LKB), and centrifuged at room temperature for 5 min at 1,000 rpm in an IEC PR-J centrifuge. Viable cells, which formed a layer above the Ficoll-Paque, were harvested and cultured under selection.

NIH 3T3 cells were plated at 1×10^6 to 1.5×10^6 cells per 100-mm dish 18 h before transfection. A 2-ml transfection mix contained the following, dissolved in DMEM without serum: 60 μg of Transfectam (Promega), 10 μg of plasmid DNA, and 1.5 μg of CMV-290 plasmid DNA, containing the hygromycin phosphotransferase gene (15). The dishes were rocked slowly on a rocker platform for 18 h at 37°C under 5% CO_2. After aspiration of the transfection mix, 10 ml of DMEM containing 10% calf serum was added to each dish. The cells were allowed to recover for 24 h and were then cultured in growth medium with 100 μg of hygromycin B per ml for 6 days and 200 μg of hygromycin B per ml thereafter. Pools of colonies, not clones, were then expanded for use in the serum starvation/induction experiments.

Stable transfectants, rather than transiently transfected cells, were used for three reasons: (i) they provided a consistent source of cells and eliminated variability associated with short-term transfections; (ii) large quantities of cells were available; and (iii) in transient transfections, the chimeric genes generated heterogeneous smears of mRNAs on Northern (RNA) blots (data not shown). These mRNAs presumably contained heterogeneous 5′ and/or 3′ termini. Discrete mRNAs were produced in the stably transfected cells (see Results).

NIH 3T3 cell serum starvation/induction. Exponentially growing cells were subcultured to six 150-mm tissue culture dishes. After they had reached approximately 70% confluence, the medium was aspirated, and the cells were washed twice with 15 ml of 37°C 1× phosphate-buffered saline (PBS). DMEM containing 0.5% calf serum was added, and cells were cultured for 25 h at 37°C under 7.5% CO_2. To induce the c-*fos* promoter, the low-serum medium was aspirated and replaced with DMEM containing 15% fetal calf serum.

Construction of chimeric β-globin–c-*myc* genes. To assess mRNA stability under different growth conditions, we constructed two sets of genes. One was cloned downstream from the CMV immediate-early promoter in plasmid pB108, which also contains the *Escherichia coli* hygromycin phosphotransferase gene and 112 bp of CMV 5′-UTR leader sequence necessary for optimal transcription (13) (Fig. 1, stippled rectangle). The other set was driven by the human c-*fos* promoter, to permit rapid and transient induction of c-*fos* transcription following serum addition to starved cells (14, 21, 37). A 373-bp *Pst*I fragment containing this promoter was cloned into the *Pst*I site of pGEM-3Z (Promega). Gene cassettes (see below) were cloned separately into pBS.KS.M13$^+$ (Stratagene) lacking the c-*fos* promoter. The promoter was then excised from the pGEM-3Z multiple cloning site by digestion with *Kpn*I and *Hin*dIII and was inserted into the *Kpn*I and *Hin*dIII sites located upstream of each gene cassette. The *fos*-derived mRNAs contained 14 nt from c-*fos*, 8 nt from the multiple-cloning site of pGEM-3Z (between the *Pst*I and *Hin*dIII sites), 6 nt from a *Hin*dIII linker, plus 11 nt before the true β-globin cap site.

The sequence of all hybrid junctions and PCR-generated fragments was verified by using double-stranded dideoxy T7 polymerase sequencing (U.S. Biochemicals). Translation of all mRNAs was initiated at the normal β-globin start codon and, unless otherwise noted, was terminated at either the normal β-globin or c-*myc* stop codon (Fig. 1).

(i) Glob genes. An *Nco*I-*Eco*RI fragment purified from the human β-globin gene of plasmid Hβ1S (25) was cloned into the *Nco*I and *Eco*RI sites of full-length β-globin cDNA in pSPKβ/c (kindly provided by K. Lang and R. Spritz). The resulting plasmid was then cut with *Nhe*I, and the ends were blunted with the Klenow fragment of *E. coli* DNA polymerase I. *Hin*dIII linkers (8-mer; Promega) were ligated to the blunt ends. The DNA was digested with *Hin*dIII and *Eco*RI, generating a fragment whose 5′ and 3′ ends were 17 bp upstream of the normal cap site and within β-globin exon 3, respectively. This fragment was purified from a gel and was cloned in a three-piece ligation with an *Eco*RI-*Pst*I fragment from Hβ1S, which included the β-globin 3′-UTR and 3′-flanking DNA fragment, plus pBS.KS.M13$^+$ (Stratagene) cut with *Hin*dIII and *Pst*I. The β-globin gene cassette from this plasmid, cgenglobin/pBS.KS, was cloned as a *Hin*dIII-*Pst*I fragment into pB108 cut with *Hin*dIII and *Nsi*I to generate the CMV-driven gene. The *Kpn*I-*Hin*dIII c-*fos* promoter fragment was cloned into cgenglobin/pBS.KS to create the c-*fos*-driven gene.

(ii) Glo-McMu genes. Glo-McMu chimeric genes included both of the c-*myc* instability determinants (CRD and 3′-UTR) plus additional upstream sequences. Attempts to express the full-length c-*myc* gene in transfected cells were unsuccessful, perhaps because excess c-*myc* protein was toxic (34). Glo-McMu expressed an mRNA containing most of exon 3 from the c-*myc* coding region and the entire c-*myc* 3′-UTR. (Mc

denotes the presence of c-*myc* coding sequences, and Mu denotes the presence of c-*myc* 3′ untranslated sequences.) β-Globin sequences from the cap site to the *Eco*RI site were fused in frame to human c-*myc* exon 3 sequences at the *Cla*I site with an *Eco*RI linker. Glo-McMu mRNA encoded amino acids 1 to 121 of β-globin (the total length of β-globin, including N-terminal methionine, is 147 amino acids), 2 amino acids from the linker, and residues 262 to 439 of c-*myc* (the total length of c-*myc* protein is 439 amino acids).

(iii) Glo-Mu genes. A c-*myc* genomic clone was cleaved with *Alu*I and *Eco*RI. The *Alu*I site is located 18 nt upstream of the c-*myc* termination codon (Fig. 1). An *Eco*RI linker was ligated to the *Alu*I site, and the fragment was cloned into the *Eco*RI site of the human β-globin gene. Glo-Mu mRNA encoded amino acids 1 to 121 of β-globin, 2 amino acids from the *Eco*RI linker, and the last 6 amino acids (residues 434 to 439) of c-*myc*.

(iv) Glob-Myc-Glob genes. Construction of the CMV promoter-driven plasmid has been described previously (3). Briefly, a 249-bp segment that includes the c-*myc* CRD (Fig. 1, black rectangle) was amplified by PCR and cloned in frame into *Eco*RI-cut β-globin genes driven by the CMV or c-*fos* promoter. Glob-Myc-Glob (GMG) mRNA contained 1,006 nt [without poly(A)] and encoded amino acids 1 to 121 of β-globin, c-*myc* amino acids 357 to 439 (which includes the CRD), 2 amino acids from the *Eco*RI linker, and amino acids 122 to 147 of β-globin.

(v) Glob-Stop-Myc-Glob genes. The same 249-bp c-*myc* CRD fragment used to construct the GMG gene was amplified from c-*myc* cDNA by PCR. The premature termination codon was created by including a TGA triplet in the 29-mer used as the 5′ primer (5′-GCGAATTCTGACGAACACACAACGTC TTG-3′). All subsequent cloning steps in producing Glob-Stop-Myc-Glob (G-SMG) were as described for GMG. The resulting plasmid contained a TGA stop codon at the junction of the β-globin sequence with the c-*myc* sequence, preventing translation of the c-*myc* CRD (see the diagram in Fig. 7).

(vi) Glob-GAPDH-Glob genes. To produce Glob-GAPDH-Glob (GGG) genes, a 249-bp fragment from rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA corresponding to the 83 carboxy-terminal codons was amplified from a GAPDH cDNA plasmid by PCR (12). The 5′ and 3′ primers were a 26-mer (5′-GCGAATTCGCCAAGTATGATGACA TC-3′) and a 28-mer (5′-CTCGAATTCCTCCTTGGAGGCC ATGTAG-3′), respectively. Each primer contained *Eco*RI restriction sites for cloning into the *Eco*RI site of cgenglobin/pBS.KS.

(vii) Glob-Stop-GAPDH-Glob genes. To produce Glob-Stop-GAPDH-Glob (G-SGG) genes, a 252-bp fragment identical to the rat GAPDH fragment described above was amplified by PCR with the same 3′ primer used to construct GGG and a 5′ 29-mer with the sequence 5′-GCGAATTCTGAGC CAAGTATGATGACATC-3′. The resulting GAPDH fragment contained a TGA stop codon at the 5′ junction with the β-globin sequence and at the same relative position as the stop codon in G-SMG (see Fig. 7).

In vitro mRNA decay reactions. Polysomes were isolated from spinner-flask cultures of exponentially growing cells (29, 30). Each 50-μl mRNA decay reaction mixture contained 30 μg of polysomal RNA and, when indicated, 2 μg of capped exogenous competitor RNA (see below). Reaction conditions and RNA isolation were identical to those of Bernstein et al. (3), with one modification. Significant nonspecific RNA degradation occurred in preliminary experiments with HeLa cell polysomes in the presence of competitor RNAs (data not shown). Such degradation was not observed with K562 cell polysomes under similar conditions (3; data not shown). Subsequent experiments revealed that the exogenous RNAs were activating HeLa RNase L, presumably via their duplex regions. To reduce RNase L activation, ATP was omitted from the reaction mixtures and 2 mM 2′dATP–magnesium acetate plus a 10^{-4} M concentration of the phosphorothioate analog of 2′,5′-oligo(A) (pSpSp) were added (2, 19, 20, 35). pSpSp refers to the phosphorothioate conformer analog that binds to and inhibits the 2′,5′-A-dependent RNase L and was kindly provided by Robert J. Suhadolnik (19, 20, 35). Some nonspecific RNA degradation also occurred as a result of omitting ATP, with both HeLa and K562 polysomes (see Fig. 6 and 9; data not shown). We believe that degradation in ATP-deficient reactions is unrelated to RNase L, but we chose to exclude ATP in order to repress the more potent RNase L activity. To facilitate detection of the low-abundance Glo-Mu and Glo-McMu mRNAs, several identical 50-μl reaction mixtures were incubated simultaneously and separate reaction mixtures harvested at each time point were pooled.

Isolation of cytoplasmic RNA. RNA was isolated by a method kindly suggested by Ann-Bin Shyu (32, 33). The initial steps were carried out at 4°C. Growth medium was aspirated, and cells were rinsed once with 15 ml of cold PBS, scraped into 15 ml of PBS with a disposable cell scraper, transferred to a 15-ml tube, and pelleted by centrifugation. The cells were resuspended in 1 ml of cold PBS, transferred to a 1.5-ml microcentrifuge tube, and centrifuged at 6,000 rpm (3,670 × *g*) for 2 min. The cell pellet was resuspended in 200 μl of Nonidet P-40 lysis buffer (10 mM Tris-Cl [pH 7.4] at room temperature, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% [vol/vol] Nonidet P-40) while being vortexed for 10 s at half-maximal speed. Cells were left for 10 min on ice and were further disrupted by pipetting 10 times in a Pasteur pipette followed by vortexing for 20 s at maximal speed. The nuclei were pelleted for 5 min at 6,000 rpm, and the cytoplasmic supernatant was harvested. The remaining steps were performed at room temperature. The supernatant was mixed with 200 μl of 2× PK buffer (200 mM Tris-Cl [pH 7.5], 440 mM NaCl, 25 mM EDTA, 2% [wt/vol] sodium dodecyl sulfate [SDS]) and then immediately with 200 μg of proteinase K (Boehringer Mannheim). The mixture was vortexed, incubated at 37°C for 30 min, and extracted with phenol-chloroform-isoamyl alcohol and then with chloroform-isoamyl alcohol alone. Sodium acetate (1/20 volume of 3.0 M sodium acetate [pH 6]) and 2 volumes of ethanol were added to the aqueous phase, and RNA was precipitated overnight at −20°C. The RNA was pelleted at 4°C for 15 min at 14,000 rpm in a Microfuge, rinsed with 70% ethanol, dried, dissolved in 50 μl of double-distilled H_2O, and quantitated spectrophotometrically.

Northern blot analysis. RNA was electrophoresed in a 1% agarose–2.2 M formaldehyde denaturing gel buffered in 40 mM morpholinepropanesulfonic acid (MOPS; pH 7), 10 mM sodium acetate, and 1 mM EDTA (26). Following capillary transfer to Zeta-Probe membranes (Bio-Rad) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), RNA was immobilized by baking at 80°C in vacuo for 2 h or by cross-linking with a Stratalinker UV Crosslinker 2400 at 120,000 μJ/cm^2 (Stratagene). The blot was prehybridized for 4 h at 42°C in 45% formamide–10× Denhardt's solution (1× Denhardt's solution is 0.02% [wt/vol] each bovine serum albumin, Ficoll, and polyvinylpyrrolidone)–1% SDS–5× SSC–50 mM Na_2HPO_4 (pH 6.7)–500 μg of denatured salmon sperm DNA per ml. Hybridization was carried out for 18 to 22 h at 42°C in 50% formamide–2× Denhardt's solution–1% SDS–5× SSC–25 mM Na_2HPO_4 (pH 6.7)–250 μg of salmon sperm DNA per ml. Blots were washed once for 15 min in 2×

SSC–0.1% SDS at room temperature, once for 15 min in 0.1× SSC–0.1% SDS at room temperature, and once for 15 min in 0.1× SSC–0.1% SDS at 55°C. [^{32}P]DNA probes were prepared by random priming (11) and included the *Hin*dIII-*Xho*I Glo-McMu fragment, which hybridizes to both the endogenous c-*myc* and all the chimeric β-globin/*myc* mRNAs; a 5′ β-globin *Hin*dIII-*Eco*RI cDNA fragment, which hybridizes only to β- and γ-globin sequences; and a *Pst*I fragment containing full-length rat GAPDH cDNA, which cross-hybridizes with human GAPDH. Radioactivity in each RNA band was quantitated with a Betascope 603 Blot Analyzer (Betagen Corp.).

Synthesis of competitor RNAs. Construction of the 182-bp c-*myc* CRD DNA fragment from position 1705 to 1886 and transcription with SP6 RNA polymerase (Promega) were carried out as previously described (3). The resulting CRD competitor RNA contained 182 nt. A 238-nt nonspecific competitor RNA was transcribed with SP6 polymerase from pGEM-3Z (Promega) plasmid DNA linearized with *Nar*I.

RESULTS

Intracellular half-lives of chimeric mRNAs. Chimeric mRNAs contained one or both putative c-*myc* mRNA instability determinants (CRD and 3′-UTR) located within human β-globin mRNA (Fig. 1). Globin mRNA was considered appropriate for these studies for two reasons. First, the half-life of human β-globin mRNA is 20 to 29 h in erythroid cells (31). Therefore, instability determinants from c-*myc* should induce measurable destabilization of a globin-*myc* hybrid mRNA, assuming that the globin sequences do not repress c-*myc* destabilization signals. Second, since HeLa and 3T3 cells lack endogenous globin mRNA, cross-hybridization of globin probes to the chimeric mRNAs is avoided. Each chimeric mRNA maintained the normal β-globin, c-*myc*, or GAPDH open reading frames and used the normal β-globin initiation codon as well as the normal β-globin or c-*myc* termination codon (Fig. 1).

We first compared the steady-state levels of the chimeric mRNAs in growing HeLa cells. mRNA levels should reflect relative mRNA half-lives, assuming that the average gene copy number per cell and the transcription rate per gene were similar in each pool, both reasonable assumptions. Each transfected gene expressed an mRNA of the predicted size (Fig. 2), and their relative steady-state levels were as follows: GGG > globin > GMG > Glo-McMu > Glo-Mu (Fig. 2 legend). On the basis of these ratios, the half-lives of Glo-McMu and Glo-Mu mRNAs were 10- to 20-fold shorter than those of GGG or globin mRNAs; the GMG mRNA half-life was approximately 3-fold shorter than that of GGG.

mRNA half-lives in HeLa cells were assessed directly by blocking transcription with actinomycin D (5 μg/ml), harvesting cytoplasmic RNA at different times thereafter, and measuring mRNA levels (Fig. 3; Table 1). The actinomycin data were consistent with the steady-state measurements. Glo-Mu and Glo-McMu mRNAs were approximately fivefold less stable than GGG mRNA, whereas GMG was threefold less stable than GGG. It is unclear why globin and GGG mRNAs had similar steady-state levels whereas globin mRNA was threefold less stable than GGG mRNA in the presence of actinomycin D. Actinomycin D often has unexpected, unexplained effects on mRNA stability (16, 28), presumably as a result of its toxicity.

To avoid possible drug effects on mRNA stability, we also measured mRNA half-lives following serum induction of the c-*fos* promoter-driven genes. Unstable mRNAs under c-*fos* promoter control are scarce in serum-starved 3T3 cells, because the c-*fos* promoter is silent and the mRNA is short-lived (14, 18, 21, 32, 33, 38). Addition of serum transiently activates the promoter, causing a burst of transcription lasting only 30 to 60 min. The level of the mRNA then increases, and its subsequent rate of disappearance reflects its half-life (18, 32, 33, 38).

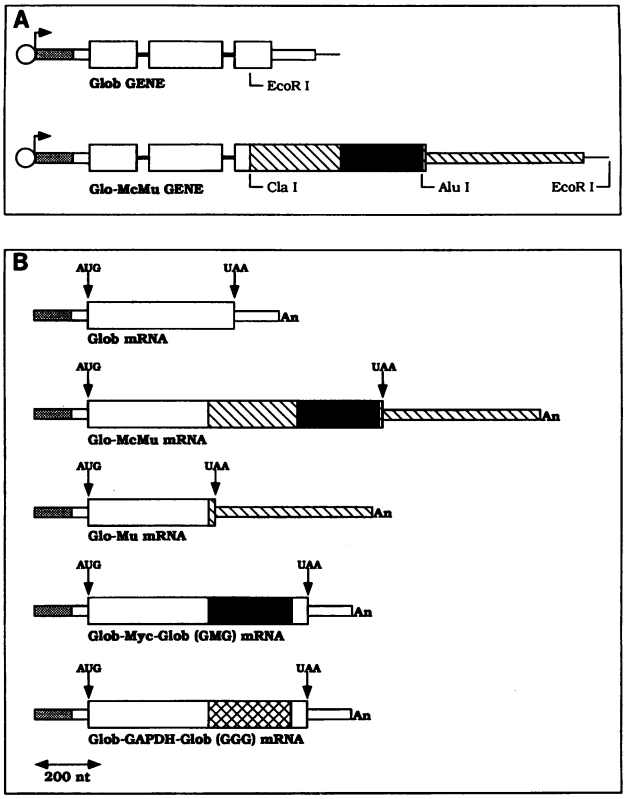

FIG. 1. Diagrams of chimeric genes and mRNAs. (A) Two of the genomic constructs are shown, to indicate important restriction sites and other features. Symbols: small rectangles, untranslated regions; large rectangles, coding regions; thick and thin lines, introns and 3′-flanking sequences, respectively; circles and arrows, the promoter. Exons, but not introns or flanking sequences, are drawn to scale. CMV and human β-globin sequences are indicated by stippled and open rectangles, respectively. Human c-*myc* sequences are indicated by the hatched rectangles, and the black rectangle indicates the c-*myc* CRD. Transcription is driven by either the human CMV immediate-early promoter or the human c-*fos* promoter. CMV-driven genes, which are diagrammed in the figure, generate mRNAs with a CMV 5′ untranslated region segment (stippled rectangle). mRNAs from *fos*-driven genes lack the CMV segment. Mc indicates the presence of some of the c-*myc* coding region, while Mu denotes the c-*myc* 3′-untranslated region. (B) mRNAs from the CMV promoter contained 122 nt derived from CMV 5′ untranslated sequences (stippled rectangles). Otherwise, each pair of CMV- and *fos*-derived mRNAs was identical. Each contained β-globin sequences extending from the β-globin 5′ terminus to the β-globin *Eco*RI site. Sequences downstream from the *Eco*RI site were from globin (open rectangles), c-*myc* (hatched and black rectangles), GAPDH (cross-hatched rectangles), or a combination of sequences. The DNA sequence of each junction was confirmed by using double-stranded sequencing. Each chimeric mRNA was translated in frame from the indicated AUG to UAA sites.

The relatively stable mRNAs generated from *fos*–β-globin and *fos*-GGG genes were detectable in NIH 3T3 cells prior to serum induction (Fig. 4A, time zero). These mRNAs were probably synthesized prior to serum depletion and persisted

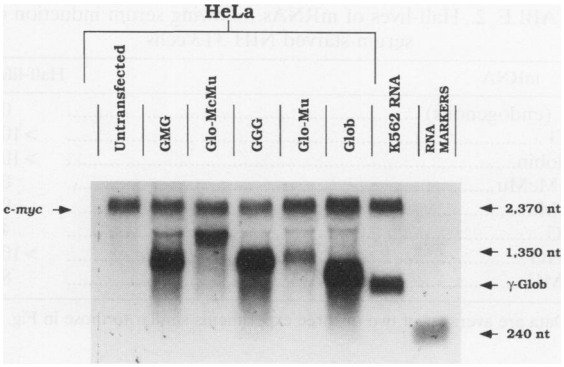

FIG. 2. Relative expression of mRNAs in transfected cells. HeLa cells were transfected with the indicated gene (Fig. 1), and pools of cells were selected in hygromycin. Cytoplasmic RNA (15 μg) was prepared from exponentially growing cells and from untransfected HeLa or K562 (human erythroleukemia) cells. The RNAs were electrophoresed in a 1% agarose–2.2 M formaldehyde denaturing gel, blotted, and detected by hybridization to a radiolabeled *Hin*dIII-*Xho*I fragment from the Glo-McMu gene (Fig. 1) that contained both globin and *myc* sequences. Band intensities were quantitated with a Betascope 603 Blot Analyzer. The relative levels of each mRNA were calculated from the amounts of endogenous c-*myc* and GAPDH (not shown) mRNAs in each lane and the percentage of each mRNA that was homologous to the probe. Two experiments were performed with separate isolates of cell RNA, and a typical result is shown. Setting the GGG mRNA level at 1, the average levels of the other mRNAs were as follows: globin, 0.80; GMG, 0.30; Glo-McMu, 0.10; Glo-Mu, 0.05. Arrows indicate the positions of the endogenous c-*myc* and γ-globin mRNAs and phage lambda RNA markers (GIBCO BRL), which were identified by hybridizing the blot with lambda [^{32}P]DNA cut with *Hin*dIII.

through the starvation period. The kinetics of mRNA appearance and decay following serum induction confirmed their stability (Fig. 4A; Table 2). mRNA levels reached their maximum by 4 h, increased by less than threefold relative to time zero, and remained elevated during the subsequent 4 h. Therefore, the precise half-lives of the mRNAs could not be determined but were longer than 10 h (Table 2).

The induction and disappearance of the globin-*myc* mRNAs confirmed their instability (Fig. 4B to D and 5; Table 2). Glo-McMu, Glo-Mu, and GMG mRNAs were less abundant at time zero than β-globin or GGG mRNAs, were induced to a greater extent (relative to time zero), and disappeared with apparent half-lives at least 2- to 20-fold shorter. Each mRNA also reached its peak level faster than the stable mRNAs did.

The following conclusions can be drawn from these experiments. (i) Each c-*myc* instability determinant functioned independently, even when embedded within a very stable mRNA. The Glo-Mu data confirm that the c-*myc* 3′-UTR contains a destabilizing sequence (1, 4, 17, 22, 24, 39). (ii) The c-*myc* CRD does influence mRNA stability in intact cells, because GMG mRNA was two- to threefold less stable than GGG mRNA. These data confirm previous results obtained with transfected myoblasts (39, 40) and with cell extracts (3). (iii) Glo-Mu mRNA was as unstable as Glo-McMu mRNA (Tables 1 and 2).

The CRD, but not the 3′-UTR determinant, is affected by the CRD competitor RNA in vitro. c-*myc* mRNA is degraded by different pathways under different conditions in intact cells (36) and in cell extracts (3, 6). In vitro experiments with exogenous CRD competitor RNA implied that the CRD-BP binds to the c-*myc* CRD and protects it from endo-RNase

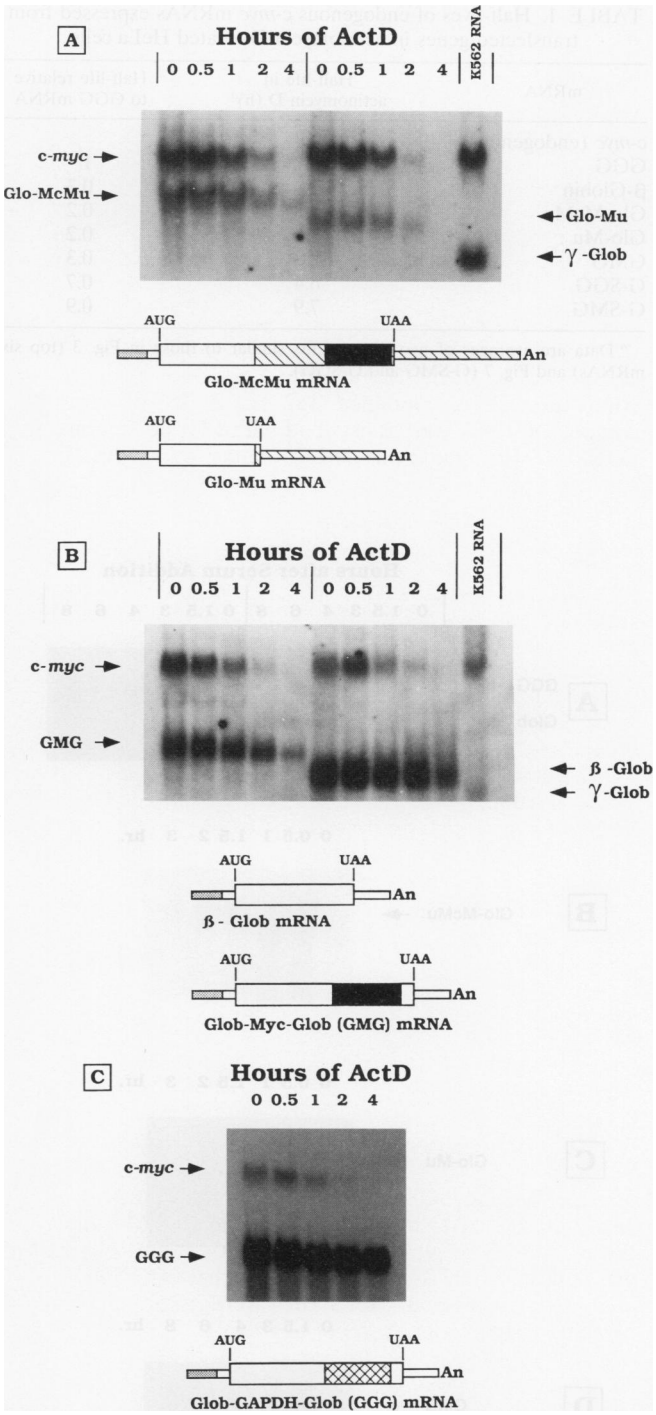

FIG. 3. Half-lives of endogenous c-*myc* and chimeric mRNAs in actinomycin D-treated HeLa cells. Cytoplasmic RNA was isolated from individual 100-mm dishes of exponentially growing, stably transfected HeLa cells exposed for the indicated times to actinomycin D (ActD; 5 μg/ml). RNAs were analyzed by Northern blotting and hybridization with radiolabeled Glo-McMu probe, as in Fig. 2. Arrows indicate the positions of endogenous c-*myc* and, in K562 cells, γ-globin mRNAs and of each chimeric mRNA. Band intensities were measured with the Betascope, and half-life data from two experiments are summarized in Table 1. (A) HeLa cells expressing Glo-McMu (lanes 1 to 5) or Glo-Mu (lanes 6 to 10) mRNA. (B) HeLa cells expressing GMG (lanes 1 to 5) or β-globin (β-Glob; lanes 6 to 10) mRNA. (C) HeLa cells expressing GGG mRNA.

TABLE 1. Half-lives of endogenous c-*myc* mRNAs expressed from transfected genes in actinomycin D-treated HeLa cells

mRNA	Half-life in actinomycin D (h)[a]	Half-life relative to GGG mRNA
c-*myc* (endogenous)	0.8	
GGG	8.7	1
β-Globin	2.8	0.3
Glo-McMu	1.8	0.2
Glo-Mu	1.5	0.2
GMG	2.8	0.3
G-SGG	6.4	0.7
G-SMG	7.9	0.9

[a] Data are averages of two experiments similar to those in Fig. 3 (top six mRNAs) and Fig. 7 (G-SMG and G-SGG).

TABLE 2. Half-lives of mRNAs following serum induction of serum-starved NIH 3T3 cells

mRNA	Half-life (h)[a]
c-*fos* (endogenous)	0.5
GGG	>10
β-Globin	>10
Glo-McMu	0.5
Glo-Mu	0.9
GMG	4.5
G-SGG	>10
G-SMG	8.0

[a] Data are averages of two or three experiments similar to those in Fig. 4 and 7.

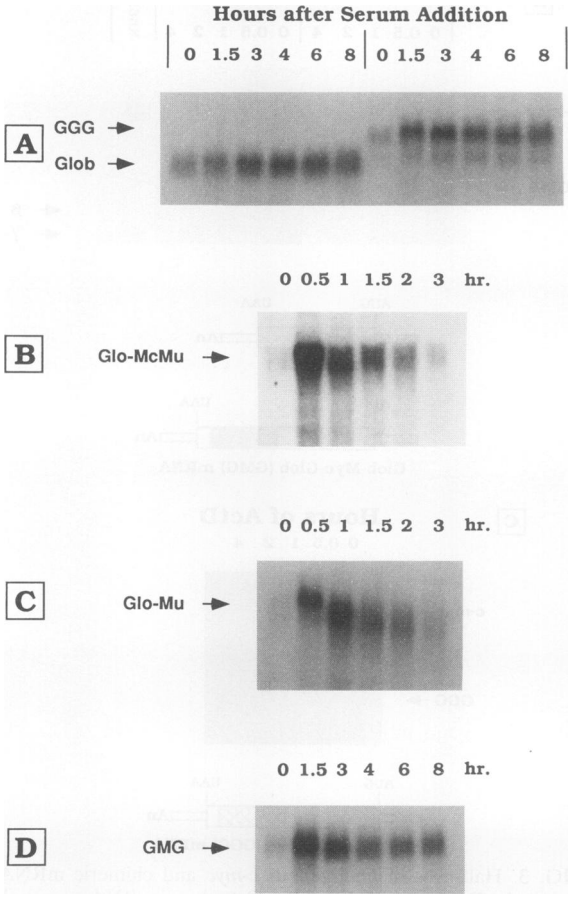

FIG. 4. Kinetics of expression of globin-*myc* and globin-GAPDH chimeric mRNAs in serum-induced NIH 3T3 cells. Stable pools of NIH 3T3 cells transfected with the indicated *fos*-driven genes were cultured in separate dishes for 25 h in medium containing 0.5% serum. The medium was then aspirated and replaced with medium containing 15% serum. At the indicated times thereafter, cells from individual dishes were harvested, cytoplasmic RNA was extracted, and 10 μg of RNA was analyzed by Northern blotting and hybridization with a *Hin*dIII-*Eco*RI β-globin cDNA probe. (A) Cells containing the β-globin (Glob; lanes 1 to 6) or GGG (lanes 7 to 12) gene. (B) Cells containing the Glo-McMu gene. (C) Cells containing the Glo-Mu gene. (D) Cells containing the GMG gene.

attack. If so, the RNA competitor should not destabilize Glo-Mu mRNA, which lacks the CRD. Polysomes containing Glo-Mu or GMG mRNA were incubated in mRNA decay reactions with or without the competitor. Some nonspecific degradation was observed in reactions lacking exogenous RNA, probably because ATP was omitted (Fig. 6 and data not shown) (see Materials and Methods). RNA from the pGEM vector induced a two- to threefold further destabilization of all the mRNAs, presumably via residual RNase L that was not

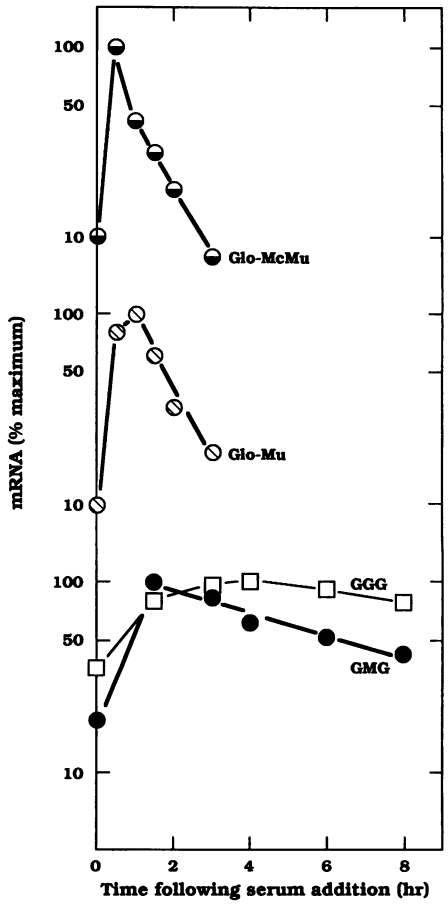

FIG. 5. Serum-induced expression of globin-*myc* and globin-GAPDH chimeric mRNAs in NIH 3T3 cells. The intensities of gel bands from Fig. 4 and from two or three other experiments were quantitated with the Betascope and averaged, and data were plotted as a percentage of the maximum mRNA level following serum induction.

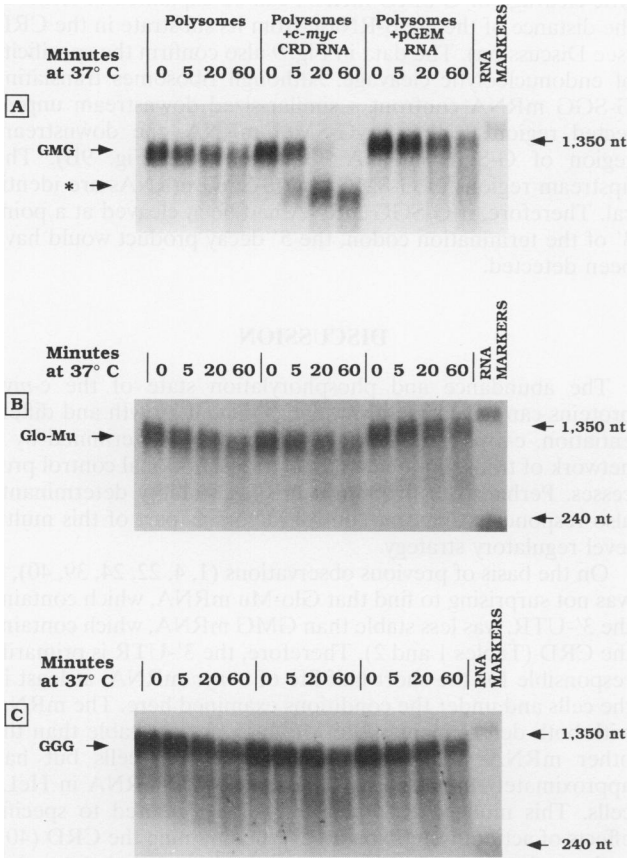

FIG. 6. Endonucleolytic cleavage of mRNA containing the c-*myc* CRD but not the 3′-UTR in cell-free mRNA decay reaction mixtures supplemented with c-*myc* competitor RNA. Polysomes prepared from HeLa cells expressing GMG (A), Glo-Mu (B), or GGG (C) mRNA were incubated in mRNA decay reaction mixtures, some of which were supplemented with 2 μg (~10^4-fold excess over mRNA) of either of two in vitro synthesized RNAs. CRD competitor RNA contained sequences from the c-*myc* CRD; pGEM RNA was from the pGEM vector. Modified reaction conditions designed to reduce RNase L activation were used (Materials and Methods). Reactions were stopped at the indicated times, and RNA was extracted and analyzed by Northern blotting with a ^{32}P-labeled Glo-McMu fragment probe (Fig. 2). Band intensities were quantitated on the Betascope, and half-lives were averaged from two experiments (Table 3). The asterisk denotes the endonuclease decay product (3).

blocked by the added RNase L inhibitors (Fig. 6; Table 3) (see Materials and Methods). However, only GMG mRNA, containing the c-*myc* CRD, was further destabilized three- to fourfold when c-*myc* CRD competitor RNA was added (Fig. 6A). GMG mRNA half-lives averaged 110, 50, and 15 min in reactions with no added RNA, pGEM RNA, or CRD competitor RNA, respectively. Glo-Mu mRNA half-lives were 125, 85, and 65 min in parallel reactions. Moreover, an endonuclease decay product was observed only in reaction mixtures containing GMG mRNA plus CRD competitor RNA (Fig. 6A, asterisk). Therefore, destabilization and susceptibility to endonuclease cleavage are properties of mRNA containing the c-*myc* CRD, not the c-*myc* 3′-UTR, even though the 3′-UTR influences the mRNA half-life in cells. The data are consistent with alternative c-*myc* mRNA decay pathways, one involving poly(A) shortening and subsequent 3′-to-5′ decay (6, 23, 36) and the other involving endonucleolytic cleavage within the

TABLE 3. Half-lives of mRNAs in cell-free mRNA decay reactions with and without c-*myc* or vector competitor RNA

mRNA	Half-life (h)[a] with:		
	No exogenous RNA	c-*myc* competitor[b]	pGEM competitor[b]
GGG	2.0	1.1 (1.8)	0.7 (2.9)
GMG	1.8	0.2 (9.0)	0.8 (2.2)
Glo-Mu	2.1	1.1 (1.9)	1.4 (1.5)
G-SGG	1.4	0.8 (1.8)	0.8 (1.8)
G-SMG	3.0	0.9 (3.3)	1.4 (2.1)

[a] Data are averages of two experiments, as in Fig. 6 and 9.
[b] Numbers in parentheses indicate the half-life ratio (no RNA/exogenous RNA) as a measure of RNA-induced destabilization.

CRD (3). In this context, it seems relevant that Glo-Mu and GMG mRNAs appeared to be degraded by different decay pathways in serum-stimulated NIH 3T3 cells (Fig. 4C and D). We do not understand why the in vitro half-life of GGG mRNA is approximately the same as that of GMG and Glo-Mu in reactions without exogenous RNA (Table 3). It might be necessary to rid the system completely of RNase L activity without omitting ATP in order to detect half-life differences in reactions with HeLa cell polysomes.

Characterization of the c-*myc* CRD: role of CRD translation in regulating mRNA stability. Since the in vitro mRNA decay system used in these experiments synthesizes little or no protein (30), it is not useful for investigating how the mechanics of translation (initiation, elongation, and termination) influence mRNA stability. However, since translation of the CRD is obviously required to generate full-length c-*myc* protein, translation through the CRD might influence the efficiency with which the CRD-BP protects the mRNA from endonuclease attack. If so, blocking ribosome translocation through the CRD should affect the mRNA half-life.

To test this possibility, two modified chimeric mRNAs were stably expressed in HeLa cells. G-SMG and G-SGG each contain a translation termination codon located 5′ of their respective inserted segments (Fig. 7). When each mRNA and its unmodified counterpart were synthesized in vitro and translated in a rabbit reticulocyte extract, protein products of the predicted sizes were produced, indicating that the premature termination codons were functional (data not shown). The half-life of G-SMG mRNA in actinomycin-treated HeLa cells was approximately 8 h, compared with 3 h for GMG mRNA (Fig. 7; Table 1). The half-lives of G-SGG and GGG were approximately 6 and 9 h, respectively. Parallel results were observed in serum-induced NIH 3T3 cells, in which G-SMG mRNA was more stable than GMG mRNA whereas the half-lives of GGG and G-SGG mRNAs were over 10 h (Fig. 8; Table 2). Therefore, blocking ribosome movement through the c-*myc* CRD prolonged the G-SMG mRNA half-life by approximately twofold in both cell types, even though the endonuclease cleavage site was unchanged (see also references 32 and 39).

To assess whether the premature termination codon affected endonucleolytic cleavage in vitro, polysomes from HeLa cells expressing the prematurely terminated mRNAs were incubated with or without the CRD or pGEM competitor RNA. Two differences were observed between GMG and G-SMG mRNAs (Fig. 9A; Table 3): (i) G-SMG mRNA was only 1.6-fold less stable (3.3/2.1) with the CRD competitor than with the pGEM competitor, whereas GMG mRNA was 4-fold less stable (9.0/2.2); and (ii) although the CRD competitor did induce some endonucleolytic cleavage of G-SMG mRNA, the

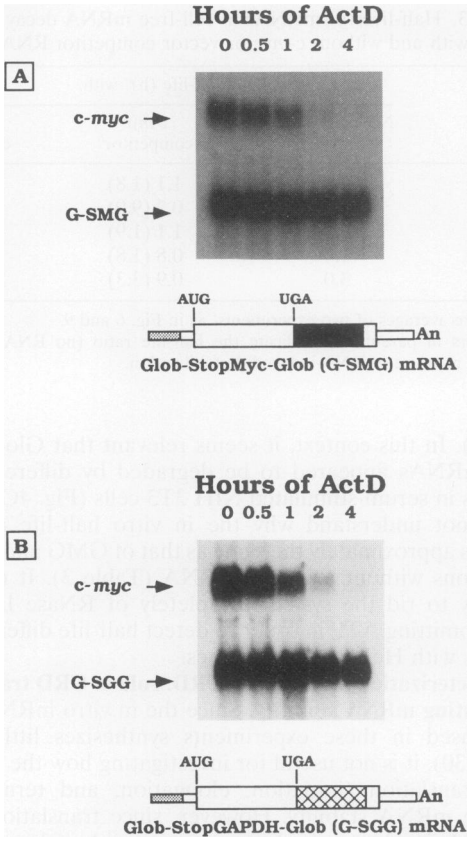

FIG. 7. Effect of premature translational termination on GMG mRNA half-life in actinomycin D-treated HeLa cells. Exponentially growing HeLa cells expressing G-SMG (A) or G-SGG (B) mRNA were exposed to actinomycin D (ActD; 5 μg/ml) for the indicated times. Cytoplasmic RNA was isolated and analyzed by Northern blotting and hybridization with the Glo-McMu probe (Fig. 2). The positions of endogenous c-*myc* mRNA and of each chimeric mRNA are indicated.

rate of cleavage was at least twofold lower than that observed with GMG mRNA (compare Fig. 6A and 9A). These data imply that ribosomes must translocate through the CRD to elicit an efficient response to the CRD competitor and to effect maximal destabilization. We suggest, but have not proved, that the endo-RNase is associated with ribosomes. If so, the modest 1.6-fold destabilization and the slower than usual endonucleolytic cleavage of G-SMG mRNA can be explained in terms of the distance of the endo-RNase from its substrate in the CRD (see Discussion). The data in Fig. 9 also confirm the specificity of endonucleolytic cleavage. Although ribosomes translating G-SGG mRNA confront a similar-sized downstream unprotected region to that in G-SMG mRNA, the downstream region of G-SGG mRNA is not cleaved (Fig. 9B). The upstream regions of G-SMG and G-SGG mRNAs are identical. Therefore, if G-SGG mRNA had been cleaved at a point 3′ of the termination codon, the 5′ decay product would have been detected.

DISCUSSION

The abundance and phosphorylation state of the c-*myc* proteins can have significant effects on cell growth and differentiation. c-*myc* protein levels, in turn, are determined by a network of transcriptional and posttranscriptional control processes. Perhaps the two c-*myc* mRNA stability determinants also respond to different control factors as part of this multilevel regulatory strategy.

On the basis of previous observations (1, 4, 22, 24, 39, 40), it was not surprising to find that Glo-Mu mRNA, which contains the 3′-UTR, was less stable than GMG mRNA, which contains the CRD (Tables 1 and 2). Therefore, the 3′-UTR is primarily responsible for the short half-life of c-*myc* mRNA, at least in the cells and under the conditions examined here. The mRNA with both determinants (Glo-McMu) was less stable than the other mRNAs in serum-induced NIH 3T3 cells but had approximately the same half-life as Glo-Mu mRNA in HeLa cells. This modest discrepancy might be related to specific effects of actinomycin D on mRNAs containing the CRD (40), and we have not further investigated potential synergism between the two determinants.

GMG mRNA was consistently less stable than GGG mRNA in mouse and human cells, confirming that the c-*myc* CRD is an mRNA-destabilizing element (40). The GAPDH segment could contain an mRNA-stabilizing sequence that specifically prolongs the GGG mRNA half-life and thereby makes GMG mRNA only seem relatively unstable. However, this notion seems unlikely for two reasons. First, endogenous GAPDH mRNA is not more stable than globin mRNA. Second, although GGG mRNA is at least twice as stable as β-globin mRNA in actinomycin-treated HeLa cells, GMG mRNA was still less stable than β-globin or GGG mRNA (Fig. 2; Table 2). GMG and β-globin mRNAs had similar half-lives in actinomycin-treated HeLa cells, but this result must be interpreted with caution because actinomycin seems to interfere with the

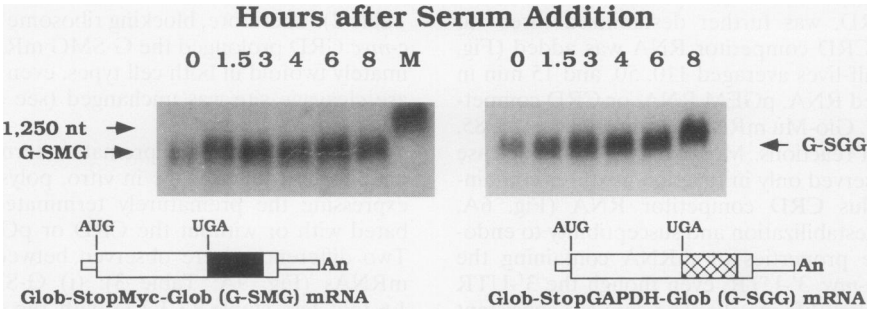

FIG. 8. Effect of premature translational termination on mRNA half-lives in serum-stimulated NIH 3T3 cells. Serum-starved NIH 3T3 cells expressing G-SMG or G-SGG mRNA were exposed to serum. RNA isolated at the indicated times was analyzed by Northern blotting and hybridization with the β-globin cDNA probe. Lane M, RNA markers; one (1,250 nt) is present in the portion of the gel shown.

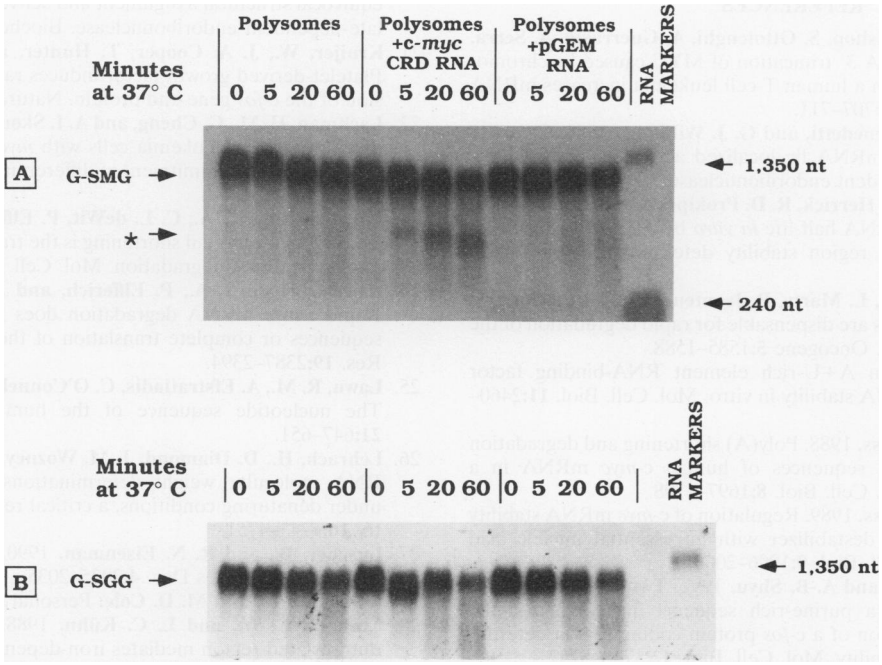

FIG. 9. Effect of c-*myc* competitor RNA on endonucleolytic cleavage of polysome-associated mRNAs containing premature translation termination codons. Polysomes isolated from stably transfected HeLa cells expressing G-SMG (A) or G-SGG (B) mRNA were incubated in reaction mixtures supplemented with no added RNA (polysomes alone), with the c-*myc* CRD competitor, or with control pGEM competitor RNA. Total RNA was isolated, blotted, and hybridized with a *Hin*dIII-*Eco*RI β-globin cDNA probe that anneals to globin sequences from the 5′ terminus to the premature termination site but does not anneal to the 3′ region of the mRNAs (see maps in Fig. 8). The asterisk denotes the endonuclease decay product (3).

decay of mRNA containing the c-*myc* CRD (40). Therefore, taken together, the results with intact cells demonstrated a destabilization sequence in the c-*myc* CRD, relative to the GGG and β-globin controls.

Perhaps the major question raised by these experiments concerns whether each stability determinant has a distinct function and responds to particular cell growth conditions. The CRD of c-*fos* mRNA influences the mRNA half-life during the serum-induced transition from stationary to growing phase but might have minimal effect on stability in exponentially growing cells (32). RNA-binding proteins with specificity for the c-*fos* coding or 3′-UTR determinants have been identified and might regulate stability under different conditions (8, 41). Similarly, one or more cytosolic factors might bind to CRD RNA sequences and protect the mRNA from a ribosome-associated endonuclease. If this or a related model is correct, it will be especially important to understand how the affinity of the CRD-BP for the mRNA is regulated in cells.

It is also necessary to determine how translation (ribosome translocation) affects the affinity of the protein for the CRD. Wisdom and Lee (40) found that mutation of the translation start codon prevented induction of c-*myc* mRNA by cycloheximide. They concluded that a CRD-containing mRNA is least stable when it is translated, which is consistent with our results. We have not excluded a possible destabilization function for the peptide encoded by the c-*myc* CRD. However, out-of-frame translation of the c-*fos* mRNA coding region does not lead to mRNA stabilization, suggesting that the primary RNA sequence, not the c-*fos* peptide, is important (38). G-SMG mRNA was more stable than GMG mRNA in cells (Fig. 7 and 8; Tables 1 and 2) and was cleaved less efficiently in cell-free reactions containing the CRD competitor (Fig. 9). We do not fully understand why G-SMG mRNA was cleaved at all in these reactions, but several explanations are feasible. If the putative endonuclease is bound to ribosomes rather than mRNA, efficient cleavage might occur only when the ribosome is translating the CRD, because translation would juxtapose the enzyme with its substrate. G-SMG mRNA would be cleaved less efficiently because the endonuclease never enters the CRD. Inefficient cleavage of G-SMG mRNA could occur by several mechanisms. The protein-free CRD segment might fold back randomly, occasionally coming within reach of the endonuclease on an upstream ribosome. Alternatively, an endonuclease on another polysome might, by chance, encounter the CRD and cleave it (a *trans* cleavage event). Perhaps these models can be tested by moving the termination site closer to the 3′ end, thus placing the endonuclease closer to its putative substrate. Understanding the cleavage mechanism will be important in determining the role of the CRD, if any, on c-*myc* mRNA stability in intact cells.

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