Control of *c-myc* mRNA half-life in vitro by a protein capable of binding to a coding region stability determinant

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Polysome-associated *c-myc* mRNA is degraded relatively rapidly in cells and in an in vitro mRNA decay system containing extracts from cultured mammalian cells. Using this system, a competition/screening assay was devised to search for factors that bind to specific regions of polysome-associated *c-myc* mRNA and thereby alter its half-life. mRNA stability was first assayed in reactions containing exogenous competitor RNAs corresponding to portions of *c-myc* mRNA itself. The addition of a 182-nucleotide sense strand fragment from the carboxy-terminal portion of the *c-myc*-coding region destabilized *c-myc* mRNA by at least eightfold. This RNA fragment had no effect on the stability of other mRNAs tested. Moreover, *c-myc* mRNA was not destabilized in reactions containing unrelated competitor RNAs or sense strand RNA from the *c-myc* 5' region. Polysome-associated globin mRNA containing the *c-myc*-coding region segment in-frame was also destabilized in vitro by the 182-nucleotide RNA. As determined by UV-cross-linking experiments, the 182-nucleotide RNA fragment was recognized by and bound to an ~75-kD polysome-associated protein. On the basis of these data plus Northern blotting analyses of *c-myc* mRNA decay products, we suggest that the ~75-kD protein is normally bound to a *c-myc*-coding region determinant and protects that region of the mRNA from endonuclease attack. Possible links between the protective protein, translation, ribosome pausing, and *c-myc* mRNA turnover are discussed.

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Three observations indicate the extent to which mRNA stability influences gene expression, especially in cells of higher organisms (Marzluff and Pandey 1988; Cleveland and Yen 1989; Peltz et al. 1991). First, although the steady-state level of any mRNA reflects its synthesis and decay rates, the half-lives of many mRNAs seem to be the major determinants of their abundance. That is, mRNA abundance often correlates more closely with how long the mRNA persists in the cytoplasm than with how rapidly it is synthesized. Second, the half-life of a mRNA sets the time required to reach a new steady state following a change in transcription rate. The shorter the half-life, the sooner the new steady state is reached (Hargrove and Schmidt 1989). Thus, a fivefold increase in the transcription of a given gene will eventually yield a fivefold increase in the abundance of the mRNA; however, a labile mRNA will reach the new steady state faster than a stable mRNA, illustrating a poorly appreciated but important link between mRNA stability and transcription. Third, as cells divide and differentiate, they modify the half-lives of specific mRNAs. For example, histone mRNA is approximately fourfold less stable in G₂ than in mid-S-phase of the cell cycle (Marzluff and Pandey 1989; Harris et al. 1991), whereas *c-myc* mRNA is stabilized four- to sixfold in response to growth factors and

translational inhibitors (Kindy et al. 1986; Brewer and Ross 1989).

The *c-myc* mRNA half-life can increase or decrease, depending on the extracellular environment. Such fluctuations probably reflect the actions of different cytoplasmic regulatory factors that become activated or repressed in response to specific environmental signals. Each factor, in turn, probably recognizes distinct mRNA stability determinants, consistent with the observation that separate regions within the mRNA molecule influence its half-life. One stability determinant is located within the 3'-untranslated region (3'-UTR), and another in the coding region (Lachman et al. 1986; Jones and Cole 1987; Bonnieu et al. 1988, 1990; Wisdom and Lee 1990, 1991; Laird-Offringa et al. 1991; this paper). Other mRNAs, including *c-fos*, also contain at least two distinct regions that influence their half-lives (Rahmsdorf et al. 1987; Shyu et al. 1989, 1990). If the overall half-life of *c-myc* mRNA is determined by two or more independently functioning, structurally unique sequence elements, then each element might interact with different *trans*-acting regulatory factors. If so, a number of questions arise. Is each regulatory factor activated only under specific circumstances, for example, during the transition from quiescence to growth, during a single phase of

cell differentiation, or following neoplastic transformation (Schuler and Cole 1988)? How does each factor interact with each region of the mRNA? What regulates the regulators?

We and others are exploiting in vitro assays to identify factors that regulate cytoplasmic mRNA stability and to determine whether these factors interact with specific regions of the mRNAs that they affect. A constitutively expressed cytosolic factor destabilizes *c-myc* and, perhaps, also *c-myb* mRNA. Inactivation of this destabilizer might account for the two- to fourfold accumulation of *c-myc* mRNA in cycloheximide-treated cells (Brewer and Ross 1989). Two proteins of 37 and 40 kD bind to AU-rich sequences located in the 3'-UTR of *c-myc* and other unstable mRNAs and accelerate *c-myc* mRNA decay in vitro (Brewer 1991).

Three considerations prompted us to exploit an in vitro mRNA decay system to search for additional regulatory factors that interact with *c-myc* mRNA and change its half-life. First, it is important to understand as completely as possible the link between mRNA turnover and gene expression. To do so, it seems essential to characterize in detail how the stability of at least one mRNA is regulated, including a complete description of its degradation pathways, the mRNases that degrade the molecule, and the factors that regulate its half-life. Second, *c-myc* mRNA provides an excellent subject for further study, because some details concerning its decay pathway are already known. Third, cell-free systems have been used to analyze mRNA half-lives and to identify regulatory factors. For example, *c-myc* mRNA is at least 20-fold less stable than γ -globin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs, both in cells and in vitro (Ross and Kobs 1986; Brewer and Ross 1988; J. Ross and G. Brewer, unpubl.). For the experiments described here, polysomes containing *c-myc* mRNA were isolated from a human erythroleukemia cell line and were incubated in a cell-free mRNA decay system. Reactions were supplemented with exogenous sense or antisense competitor RNA fragments corresponding to different portions of *c-myc* mRNA. If a regulatory factor were bound to a specific region of the mRNA, the factor might be titrated away by a competitor homologous to that region, thereby altering the mRNA half-life.

We find that one sense strand competitor fragment, containing the 182 carboxy-terminal-coding nucleotides, induces at least an eightfold destabilization of *c-myc* mRNA. Gel-shift and UV-cross-linking experiments reveal a ~75-kD protein that interacts with this RNA fragment. The protein sediments along with polysomes but is solubilized by exposing the polysomes to high salt. Northern blotting analyses suggest that the protein protects *c-myc* mRNA from endonucleolytic cleavage within the 182-nucleotide region, which we term the coding region determinant. In cell-free reactions, this protein also appears to protect a chimeric mRNA containing the *c-myc*-coding region determinant embedded within the translated region of human β -globin mRNA. A model is presented to suggest how the ~75-kD protein

might interact with the coding region determinant and how it might influence *c-myc* mRNA stability under specific conditions.

Results

Destabilization of c-myc mRNA in vitro by competition with sense strand RNA fragments from the carboxy-terminal-coding region

c-myc mRNA is unstable in vitro in reactions containing polysomes, which serve as a source of endogenous mRNA substrate and degradation enzymes (Ross and Kobs 1986; Brewer and Ross 1988). The short in vitro half-life reflects *c-myc* mRNA lability in intact cells (Dani et al. 1984). To determine whether the polysomes contain RNA-binding proteins that influence *c-myc* mRNA turnover, various competitor RNAs were added to cell-free reactions. If a competitor sequestered a regulatory factor, the stability of the mRNA might be altered. If the factor were specific, only *c-myc* mRNA stability should be affected by a *c-myc* competitor.

Sense and antisense *myc*-specific competitor RNAs were synthesized in vitro using the SP6 RNA polymerase promoter linked to fragments from different regions of the 2365-nucleotide *c-myc* cDNA (Fig. 1A,B). Nonspecific competitor RNAs were generated from human β -globin and pGEM vector DNAs. Each competitor was added separately to mRNA decay reactions. At various times, the incubations were stopped, RNA was isolated, and the amount of undegraded mRNA was measured by Northern blot or RNase protection analysis, using the probes indicated in Figure 1C. Sense strand competitors from the *c-myc* 3' region reduced the *c-myc* mRNA half-life by at least eightfold, from 40 min to <5 min (Fig. 1, competitors *b-d*; Figs. 2 and 3). The following RNA competitors did not destabilize *c-myc* mRNA: a *c-myc* antisense fragment from the 3' region (RNA *a*), β -globin, and pGEM RNA (Fig. 2). Therefore, the destabilization effect of RNAs *b-d* was sequence specific.

The smallest destabilizing fragment tested contained 182 nucleotides of *c-myc*-coding sequence and lacked 3'-UTR sequences (Fig. 2B, competitor *d*). On the basis of this and other observations (see below), this region of *c-myc* mRNA is referred to as the coding region stability determinant. These in vitro data are completely consistent with observations made on intact cells, implicating the coding region as an important determinant of *c-myc* mRNA stability and regulation (Lachman et al. 1986; Bonniieu et al. 1988, 1990; Wisdom and Lee 1990, 1991; Laird-Offringa et al. 1991).

To assess whether destabilization could also be induced by a sense RNA competitor from the 5' half of the mRNA, polysomes were incubated with RNA *e*. *c-myc* mRNA decay was analyzed by RNase protection with a 3' probe, to avoid hybridization of the 5' Northern blot probe to the competitor. RNA *e* had no apparent effect on *c-myc* mRNA stability (Fig. 3). This result further confirms the sequence specificity of the destabilization effect. It is also consistent with the observation that de-

Figure 1. *c-myc* mRNA competitor RNAs used in this study and summary of in vitro half-life measurements. (A) Diagram of *c-myc* mRNA. Solid lines represent 5'- and 3'-UTRs. The boxed area represents protein-coding sequences. The hatched box between nucleotides 1705 and 1886, encoding amino acids 357–439, represents the coding region determinant in the mRNA; it also denotes the smallest competitor RNA tested that induced *c-myc* mRNA destabilization (RNA *d*, see below). Restriction enzyme sites used to generate each competitor RNA are indicated above the line drawing. (B) Competitor RNAs and their effects on the half-life of polysome-associated *c-myc* mRNA in vitro. The location and orientation of each competitor RNA are indicated relative to the mRNA. Thus, RNA *a* is from nucleotides 1354–2365 and is antisense to the mRNA. Nonspecific RNAs were generated from a pGEM vector and from human β -globin cDNA. (Right) The approximate in vitro half-life of *c-myc* mRNA in reactions containing the indicated competitor RNA. These numbers represent the average of at least three experiments. (C) Probes used for detecting *c-myc* mRNA. The Northern blot probe was double-stranded DNA from the indicated region. The RNase protection probe was single-stranded RNA. The straight line indicates homologous sequence; the wavy line represents sequence from the vector.

leting most of the *c-myc* 5'-UTR (and not replacing it with other sequences) has little or no effect on *c-myc* mRNA stability in intact cells (Bonnieu et al. 1988; see also Pei and Calame 1988, whose data and conclusions differ from ours and from Bonnieu et al.)

Specificity of the coding region competitor RNAs for *c-myc* mRNA

The RNA competitors that induce destabilization derive from one region of *c-myc* mRNA. That is, sense RNAs from the coding region are effective, whereas antisense RNA from the same region and sense RNA from the 5' region are not (Figs. 1–3). To determine whether the coding region competitors affected only *c-myc* mRNA, the half-lives of four other mRNAs were analyzed. RNA *c* induced *c-myc* mRNA destabilization (Fig. 3) but had no effect on γ -globin, which is polyadenylated and stable, or on histone, which lacks poly(A) and is unstable (Fig. 4, A and B, respectively). The decay of the stable GAPDH mRNA was also unaffected by competitor RNA *c* (data not shown).

The turnover of *c-myc* mRNA was then analyzed, because, like *c-myc*, it is polyadenylated and unstable. *c-myc* mRNA half-life was approximately 23 and 32 min

without and with RNA *d*, respectively, and was therefore not significantly influenced by this competitor (Fig. 5, boxes). The turnover of AP-4 mRNA was analyzed, because both *c-myc* and AP-4 mRNAs are thought to encode transcription factors containing a three-motif segment—basic region, helix-loop-helix, and leucine zipper (Blackwell et al. 1990; Luscher and Eisenmann 1990). In this respect, AP-4 and *c-myc* mRNAs are even more closely related than *c-myc* and *c-myc* mRNAs. However, AP-4 mRNA was not destabilized in reactions containing RNA *d* (Fig. 5, circles). Its in vitro half-life was 28 min, with or without competitor. Therefore, the 182-nucleotide RNA *d* competitor does not destabilize every mRNA and shows considerable specificity for *c-myc* mRNA.

The 182-nucleotide RNA d competitor seems to expose an endonuclease-sensitive site and is effective in destabilizing a heterologous mRNA containing the myc-coding region determinant

Two observations suggest that RNA competitors *b–d* induce *c-myc* mRNA destabilization by exposing an endonuclease cleavage site within the coding region determinant. First, the asterisks in Figure 2 indicate a band (iden-

Figure 2. Effect of competitor RNAs on the in vitro decay of polysome-associated *c-myc* mRNA. (A) Competitors *a* and *b*. Standard in vitro mRNA decay reactions contained 0.7 A_{260} units of polysomes and were either unsupplemented (lanes 5–8, none) or supplemented with 1 μ g of competitor RNAs corresponding to the 1012 terminal nucleotides of *c-myc* mRNA, either in the antisense (lanes 1–4, RNA *a*) or sense (lanes 9–12, RNA *b*) orientation. Each reaction was incubated for the times indicated, RNA was extracted, and 5 μ g was electrophoresed. Following transfer to a membrane, *c-myc* mRNA levels were analyzed by hybridization with a 5' *c-myc*-specific probe (see Fig. 1C). The asterisk (*) indicates a possible *c-myc* mRNA endonucleolytic decay product (see Results and Discussion). (B) Competitor *d* (conditions were as described for A). In vitro mRNA decay reactions were supplemented with 1 μ g of either pGEM vector RNA (lanes 1–5) or competitor *d* (lanes 6–10). Lane M contains radiolabeled pBR322 DNA cleaved with *Hae*II; fragment sizes are indicated in nucleotides at left. (*) See A for explanation.

tified using a 5'-specific *c-myc* probe) with properties expected of a 5' endonuclease-generated decay product. It contains ~1800 nucleotides, consistent with a cleavage product spanning from the cap site to approximately nucleotide 1800 (see map, Fig. 1A). It was generated only in reactions containing RNA competitors *b*–*d*, which induced the destabilization effect (Fig. 2B, cf. lanes 1–5

with 6–10). As expected for a short-lived decay product, it appeared and then disappeared as the mRNA was being degraded. Second, decay products with termini in the 3'-UTR were not observed in reactions containing the destabilizing competitor *c* (Fig. 3, lanes 5–8). We have consistently detected 3' region decay products in reactions containing polysomes alone (Brewer and Ross

Figure 3. Effect of sense strand RNA competitors from the 5' and 3' regions of *c-myc* mRNA on *c-myc* mRNA stability in vitro. mRNA decay reactions were performed as described in Fig. 2. RNase protection was carried out by hybridizing 5 μ g of RNA from each time point to a uniformly labeled *c-myc* RNA probe (see Fig. 1). The four protected bands indicated by the bracket represent full-length, undegraded *c-myc* mRNAs, which terminate at four different but nearby polyadenylation sites (Brewer and Ross 1988). The half-life of the four protected bands in the presence of nonspecific RNA or RNA *e* was 60 min; the half-life with RNA *c* was 15 min. The arrowhead indicates 3' region *c-myc* mRNA decay products (Brewer and Ross 1988; see Results).

Figure 4. Effect of *c-myc* RNA competitors on γ -globin and histone mRNA stability in vitro. (A) γ -Globin mRNA. mRNA decay reactions were carried out as per Fig. 2. One microgram of RNA from each time point was analyzed by hybridization to an end-labeled β -globin 32 P-labeled DNA probe, followed by S1 nuclease digestion. The protected fragment was analyzed by electrophoresis in a 7 M urea/6% polyacrylamide gel and quantitated by scanning densitometry. The arrow indicates the band corresponding to undegraded γ -globin mRNA. Less than 10% of the mRNA was degraded during the reaction. (B) Histone mRNA. RNA (1.5 μ g) from each time point was hybridized to a 3'-end-labeled H4 histone 32 P-labeled DNA probe, followed by S1 nuclease digestion. The protected fragment was analyzed by electrophoresis in a 7 M urea/10% polyacrylamide gel. The band corresponding to the full-length protected probe is indicated by the arrow. Lower molecular weight bands are decay products. The half-life of histone mRNA was 20 min in each reaction.

1988). They arise as part of a two-step decay pathway: poly(A) shortening followed by 3' $\rightarrow$ 5' decay of the mRNA body. The 3' decay products were observed in reactions containing nonspecific RNA or RNA e (Fig. 3, lanes 1–4 and 9–12) but not in reactions containing RNA c (lanes 5–8). This result confirms that *c-myc* mRNA was degraded by a different pathway when RNA c was present. Taken together, the data from Figures 2 and 3 suggest that the destabilizing competitor RNAs function by titrating away a protective factor from the *c-myc* mRNA-coding region, inducing rapid degradation by an alternate endonucleolytic pathway.

To determine whether the *c-myc*-coding region determinant is sufficient to influence mRNA turnover and contains an endonuclease-sensitive site, we examined the effect of this region in the context of a stable mRNA molecule. The carboxy-terminal 249 *c-myc*-coding nucleotides were fused in-frame within the β -globin gene, so that the resulting chimeric mRNA would initiate and terminate translation at the usual globin sites and would encode a complete globin protein interrupted by *c-myc* residues 357–439 (amino acid 439 is at the *c-myc* carboxyl terminus; Fig. 6A). The chimeric gene was subcloned into a cytomegalovirus (CMV) promoter-driven eukaryotic expression vector, the gene was introduced into HeLa cells, and a pool of hygromycin-resistant cells synthesizing the chimeric mRNA was selected. As determined by exposing cells for 2 hr to actinomycin D and measuring mRNA levels, the half-life of the chimeric mRNA was only twofold less than that of unmodified

β -globin mRNA (data not shown). There are several possible interpretations of this observation. One is that the chimeric mRNA is unstable, but not in the presence of actinomycin D (Wisdom and Lee 1991). Another is that the *c-myc*-coding region determinant has no effect on mRNA stability in growing cells but functions only under special circumstances, for example, during cell differentiation or following drastic changes in the cell growth rate (see Discussion). In either case, the stability of the chimeric mRNA in actinomycin-treated cells is reflected in vitro in reactions lacking destabilizing competitor RNAs (Fig. 6B,C).

Polysomes were isolated from the transfected HeLa cells and incubated with or without competitor RNA d. Endogenous *c-myc* mRNA was unstable in unsupplemented reactions, indicating that in vitro reactions with HeLa cell polysomes reflect the intracellular lability of *c-myc* mRNA (Fig. 6B,C, lanes 1–4). GlobMycGlob chimeric mRNA was stable during the 1-hr time course in unsupplemented reactions (Fig. 6B, lanes 1–4). Therefore, the coding region determinant per se did not confer instability to a normally stable mRNA. RNA d addition reduced the half-life of the chimeric mRNA by at least 20-fold, to <5 min, similar to its effect on endogenous *c-myc* mRNA (Fig. 6B, lanes 5–8). Therefore, the 249-nucleotide segment contains a coding region determinant that can influence mRNA stability in vitro within the context of a stable mRNA and independent of other *c-myc* sequences.

Moreover, the 249-nucleotide region probably con-

Figure 5. Specificity of competitor RNA-induced destabilization of *c-myc* mRNA: failure to destabilize *c-myc* and AP-4 mRNAs. mRNA decay reactions, performed as described in Fig. 2, were supplemented with either pGEM RNA or competitor RNA *d*. Total RNA extracted from each reaction was analyzed by Northern blotting with probes for *c-myc*, AP-4, and *c-myc* mRNAs. The amount of undegraded mRNA remaining at each time point was quantitated by scanning densitometry. The half-lives (in min) of each mRNA without and with RNA *d*, respectively, were 23 and 32 for *c-myc*, 28 and 28 for AP-4, and 28 and <5 for *c-myc*.

tains an endonuclease cleavage site. The asterisk in Figure 6B indicates a band that appeared only in reactions with competitor RNA *d*. Its size, ~570 nucleotides, is consistent with its being a 5' decay product generated by endonuclease cleavage within the *c-myc* segment of GlobMycGlob mRNA. The possibility that the ~570-nucleotide band was a 3' decay product from endogenous *c-myc* mRNA was excluded, because the blot was stripped and rehybridized with a 5'- β -globin probe, which also detected the ~570-nucleotide band (Fig. 6C). In summary, the *c-myc*-coding region determinant can, under appropriate circumstances, influence mRNA stability in vitro without additional *c-myc* sequences, and it contains an endonuclease-sensitive site.

Identification of a protein that interacts specifically with the *c-myc* RNA competitor *d*

The data presented above are consistent with the following hypothesis: A *c-myc* mRNA-specific factor (protein) is associated with polysomes, perhaps bound to the mRNA itself. Addition of competitor RNA from the carboxy-terminal 182 nucleotides of the coding region titrates the protein away from the polysomes, leading to accelerated *c-myc* mRNA decay. To assess this possibility, gel mobility-shift assays were performed. Uniformly radiolabeled RNA *d* was incubated with polysomes in a mock mRNA decay reaction, to allow RNA-protein (RNP) complexes to form. The reaction mixture was treated with RNase T1 to digest free RNA, followed by heparin to dissociate loosely bound complexes. RNP complexes were distinguished from unbound RNA by electrophoresis in a nondenaturing gel and autoradiogra-

phy. A slower migrating RNP band was observed in reactions containing polysomes plus 32 P-labeled RNA *d* (Fig. 7A, cf. lanes 1 and 2). This band was not generated with β -globin 32 P-labeled RNA (lane 3) or when polysomes were pretreated with trypsin, indicating that the band shift depended on the interaction of a polysomal protein with 32 P-labeled RNA *d* (lane 4).

To determine whether the protein could be solubilized and separated from polysomes, as is the case with polysome-associated RNases (Ross et al. 1987), polysomes were incubated on ice with 1 M NaCl and then centrifuged, pelleting the polysomes and leaving a supernatant, termed ribosomal salt wash (RSW). When RSW was analyzed by gel-shift assay with 32 P-labeled RNA *d*, a slower migrating band was observed (Fig. 7A, lane 5). Its mobility was similar or identical to the gel-shifted band generated with polysomes (lanes 2,5), and its appearance was trypsin sensitive (lane 6). Therefore, the same protein in polysomes and RSW was probably responsible for binding to RNA *d*. This observation is important for two reasons. First, it confirms the noncovalent interaction of the protein with polysomes or *c-myc* mRNA, as evidenced by its displacement with either competitor RNA or high salt. Second, the ability to solubilize the protein in active form should facilitate its purification.

To confirm the specificity of the RNA-protein interaction, gel-shift analyses were performed by incubating polysomes with 32 P-labeled RNA *d* in the presence of unlabeled RNA. As much as 200 ng of unlabeled β -globin RNA, corresponding to a 200-fold molar excess over 32 P-labeled RNA *d*, failed to eliminate the gel-shifted band (Fig. 7B, lane 4). At 500 ng of unlabeled β -globin RNA (a 500-fold molar excess), the gel-shifted band was dimin-

Figure 6. Competitor RNA-induced destabilization of chimeric GlobMycGlob mRNA (1006 nucleotides) containing 249 nucleotides from the *c-myc* mRNA carboxy-terminal coding region. (A) Diagram of chimeric mRNA generated from the GlobMycGlob transfected gene. A 249-bp fragment from the *c-myc* gene (hatched box) was placed into the *EcoRI* site of a human β -globin gene (see Materials and methods). The *c-myc* fragment includes the entire 182 nucleotides of RNA *d* plus 67 nucleotides 5' of the RNA *d* segment. Therefore, all *c-myc* sequences are derived from the coding region (amino acids 357–439). Sequencing of the construct confirmed that the *c-myc* segment was in-frame and that translation should begin and end at β -globin initiation and termination sites, which are noted. In summary, the protein encoded by this mRNA will contain globin amino acids 1–121, followed by *c-myc* amino acids 357–439, followed by globin amino acids 122–146. The shaded boxes denote β -globin-coding sequences, the open bar denotes the 5'-UTR sequences from CMV; the solid bars indicate the β -globin untranslated regions. Methods for transfecting HeLa cells and selecting a stable pool of transformants expressing this mRNA are described in Materials and methods. (B) Destabilization by competitor *d*: analysis with a probe recognizing both globin and *c-myc* sequences. Polysomes were isolated from HeLa cells expressing the GlobMycGlob gene and were incubated in cell-free mRNA decay reactions with or without competitor RNA *d*. RNA extracted from each time point was analyzed by Northern blotting, using a hybrid gene probe containing the *HindIII-EcoRI* 5' β -globin fragment fused to the *Clal-EcoRI* fragment of the human *c-myc* gene. This probe hybridizes to GlobMycGlob and to endogenous *c-myc* mRNAs and recognizes both globin and *c-myc* sequences. Arrows denoting *c-myc* and GlobMycGlob (right) indicate the expected positions of the respective undegraded mRNAs. The sizes (in nucleotides) of DNA marker fragments coelectrophoresed are noted at left. The asterisk (*) denotes putative endonuclease decay product. (C) Destabilization by competitor *d*: analysis with a probe recognizing only 5'-globin sequences. The blot in B was stripped and reprobed with a radiolabeled DNA fragment prepared from the 5' region of the β -globin gene. The asterisk (*) denotes putative endonuclease decay product.

ished in intensity but was not eliminated (lane 5). In contrast, only 50 ng of unlabeled RNA *d* diminished the intensity of the gel-shifted band (cf. lanes 5 and 6). We conclude that the RNA-binding protein specifically recognizes *c-myc* mRNA sequences from the carboxy-terminal-coding region.

RNA-protein cross-linking by UV light was used to estimate the molecular weight of the binding proteins. Binding reactions were carried out as for the gel shifts, but reaction mixtures were exposed to UV light prior to electrophoresis, so as to link the protein covalently to the ^{32}P -labeled RNA substrate. The RNP was then digested with RNase A and analyzed by SDS-PAGE and autoradiography. A protein of ~75-kD was detected when RSW and ^{32}P -labeled RNA *d* were mixed and UV-cross-linked (Fig. 8, lane 3). Cross-linking required both RSW and UV light (cf. lane 3 with lanes 1 and 2) and was

eliminated by pretreating the RSW with trypsin (lane 4). When pGEM vector ^{32}P -labeled RNA was used instead of *c-myc* fragment *d*, the ~75-kD protein was not labeled (lanes 6,7). These results confirm the specificity of the RNA-protein interaction for *c-myc* sequences and identify a polysome-associated, ~75-kD protein responsible for binding.

Two considerations prompted us to test whether the ~75-kD protein might be poly(A)-binding protein (PABP). First, adenylate residues comprise 35% of RNA *d*, and some are clustered (Fig. 9). Second, the molecular weights of the UV-cross-linked protein and PABP are similar (Adam et al. 1986; Sachs et al. 1986; Grange et al. 1987). Yeast PABP synthesized in bacteria binds to poly(A) and affects mRNA stability in our cell-free system (Bernstein et al. 1989). However, when 1 μg of yeast PABP was mixed with polysomes and UV-cross-linked,

Figure 7. Gel-shift analyses: binding of polysome-associated protein to competitor RNA *d*. (A) Uniformly ³²P-labeled competitor RNA *d* was incubated with polysomes in a mock decay reaction (Materials and methods), and protein-RNA complex formation was assessed by electrophoresis in a 6% nondenaturing polyacrylamide gel. (Lane 1) Input ³²P-labeled RNA *d*, no polysomes; (lane 2) ³²P-labeled RNA *d* plus polysomes; (lane 3) ³²P-labeled β-globin transcript (200 nucleotides) plus polysomes; (lane 4) ³²P-labeled RNA *d* plus polysomes pretreated with trypsin; (lane 5) ³²P-labeled RNA *d* plus 1 μg of RSW protein, corresponding to material solubilized from the same number of polysomes analyzed in lane 2; (lane 6) ³²P-labeled RNA *d* plus RSW protein pretreated with trypsin. (B) Effect of unlabeled RNAs on the gel shift of ³²P-labeled RNA *d*. The amounts (μg) of each unlabeled RNA are indicated above the lanes. (Lane 1) Input ³²P-labeled RNA *d*, no polysomes; (lane 2) ³²P-labeled RNA *d* plus polysomes; (lanes 3–5) ³²P-labeled RNA *d* plus polysomes plus unlabeled β-globin RNA; (lanes 6–8) ³²P-labeled RNA *d* plus polysomes plus unlabeled RNA *d*.

the ~75-kD band was not detected (Fig. 8, lane 5). Therefore, the ~75-kD protein was probably not PABP.

Discussion

We are using in vitro assays to search for cellular proteins with two properties: the capacity to bind to a discrete region of a single polysomal mRNA and the capacity to influence the half-life of that mRNA. To screen for such proteins, sense and antisense RNAs corresponding to various segments of *c-myc* mRNA were added to a cell-free mRNA decay system containing polysomes. The half-life of *c-myc* mRNA was then compared in reactions with or without the exogenous RNA.

These assays identified a sense strand RNA fragment that reduced the half-life of *c-myc* mRNA by at least eightfold. The RNA fragment corresponded to the carboxy-terminal 182 nucleotides of the mRNA-coding region and was cross-linked by UV light to a polysomal protein of ~75 kD. The following observations indicated a considerable degree of specificity for the *c-myc* mRNA-protein interaction and for the destabilization effect: (1) Only *c-myc* mRNA was destabilized in vitro by the 182-nucleotide RNA; the half-lives of other

mRNAs were unaffected. (2) Other competitor RNA fragments, including one from the *c-myc* 5' region, failed to destabilize *c-myc* mRNA. (3) A chimeric mRNA containing 249 nucleotides from the *c-myc*-coding region, which included the 182-nucleotide sequence, was destabilized in vitro by the competitor. To account for these observations, we suggest that the 182-nucleotide RNA titrated the ~75-kD protein away from *c-myc* mRNA, exposing an endonuclease-hypersensitive site in the coding region determinant and destabilizing the mRNA (see below).

There are at least two important caveats with regard to this interpretation. First, while the ~75-kD protein sediments with polysomes, we have not demonstrated that it is actually bound to *c-myc* mRNA. However, if the protein were associated with ribosomes or with many mRNAs, rather than with a single mRNA, it would be difficult to account for the considerable specificity of the destabilization effect. Second, we have not added purified ~75-kD protein back to mRNA decay reactions and shown restoration of the 40-min half-life. Experiments are in progress to purify and characterize the protein. Two of its properties suggest that it is not related to the recently described AU-binding proteins (Malter 1989; Bohjanen et al. 1991; Brewer 1991; Malter and Hong

Figure 8. UV-cross-linking of a RSW protein to ^{32}P -labeled RNA *d*. Uniformly ^{32}P -labeled RNA *d* was incubated with 1 μg of RSW protein in a mock mRNA decay reaction and exposed to UV light (Materials and methods). Reaction mixtures were treated with RNase A, boiled in SDS-PAGE sample loading buffer, and analyzed in a 10% SDS-polyacrylamide gel. Numbers at left indicate molecular masses (kD) of coelectrophoresed protein markers. (Lane 1) No RSW; (lane 2) complete reaction containing RSW and ^{32}P -labeled RNA *d*, but no UV; (lane 3) complete reaction, plus UV; (lane 4) same as lane 3, but RSW pretreated with trypsin; (lane 5) same as lane 3, but 1 μg of PABP in place of RSW; (lanes 6,7) reaction carried out with ^{32}P -labeled pGEM RNA in place of RNA *d*, either with (lane 7) or without (lane 6) subsequent exposure to UV light.

1991; Vakalopoulou et al. 1991): (1) The sizes of the proteins differ; and (2) the carboxy-terminal-coding region of *c-myc* mRNA is purine rich but not AU rich (Fig. 9A).

Three observations indicate that *c-myc* mRNA is not degraded by the previously described two-step pathway [poly(A) shortening, followed by 3' $\rightarrow$ 5' decay (Brewer and Ross 1988)] in reactions containing destabilizing competitor RNAs. Rather, the data imply that an endonuclease cleavage site becomes exposed when the ~ 75 -kD protein is titrated away from the *c-myc*-coding region determinant. (1) A short-lived *c-myc* decay product of ~ 1800 nucleotides appears during incubations containing the destabilizing RNAs *b* and *d* (denoted by asterisks in Fig. 2). Its size corresponds to the 5' fragment generated by endonuclease cleavage within the *c-myc*-coding region determinant (nucleotides 1705–1886). (2) The ~ 1800 -nucleotide product is not observed in reactions lacking destabilizing RNAs (Fig. 2). Conversely, products of the two-step pathway (denoted by the arrows in Fig. 3, lanes 1–4 and 9–12) are observed only in reactions containing RNAs that fail to generate the ~ 1800 -nucleotide product. (3) In reactions containing competitor RNA *d*, a decay product from the GlobMycGlob chimeric mRNA is the expected size of an endonuclease cleavage product (denoted by asterisks in Fig. 6B,C).

The existence of two distinct cytoplasmic stability de-

terminants in *c-myc* mRNA, one in the 3'-UTR and another in the coding region, has been well-documented in intact cells by transfection experiments with chimeric gene constructs (Bonnieu et al. 1988; Wisdom and Lee 1991). Coding region stability determinants have also been described in *c-fos*, tubulin, and interleukin-2 (IL-2) α -receptor mRNAs (Yen et al. 1988a,b; Shyu et al. 1989, 1990; Kanamori et al. 1990). Our results are also consistent with experiments describing *trans*-acting factors that influence the half-lives of specific mRNAs in intact cells (Schuler and Cole 1988; Casey et al. 1989; Kuchka et al. 1989; Mullner et al. 1989; Stern and Grussem 1989; Weber et al. 1989; Wager and Assoian 1990; Bohjanen et al. 1991; Brewer 1991; Liang and Jost 1991; Malter and Hong 1991).

Our data suggest a model for regulation of *c-myc* mRNA stability by a protein-mRNA complex, but the nature of the complex, its precise function, and its relationship to translation, if any, are unclear. Does the ~ 75 -kD protein actually bind within the 182-nucleotide coding region determinant and protect that region from endonucleolytic attack? If so, is the protein-mRNA association influenced by *trans*-acting mRNA stability regulatory factors? If separate mRNA stability determinants exist in the *c-myc* 3'-UTR and coding region, does each function separately and respond to different regulatory factors? Does the protein remain bound while the mRNA is being translated?

For discussion purposes, we might assume that the coding region determinant, in its unprotected state, is a ready target for endonuclease digestion. However, this region consists entirely of protein-coding sequences; and, in growing cells, ribosomes might pass continuously through the region, displacing any bound proteins and perhaps blocking both the protein binding site and the endonuclease cleavage site. How, then, could the ~ 75 -kD protein protect the mRNA from decay? If ribosomes protected the site and/or blocked access to it, a protective protein would be superfluous.

On the other hand, if *c-myc* mRNA were not uniformly translated, and if ribosomes paused just upstream of or within the coding region determinant, the resulting ribosome-free segment might become an endonuclease target site (Fig. 9B). Perhaps the ~ 75 -kD protein serves to protect the mRNA from the consequences of ribosome pausing. There are many examples of ribosome pausing: while the signal recognition particle interacts with the signal sequence (for review, see Walter et al. 1984; Siegel and Walter 1988), at translation initiation and termination codons (Lindhout et al. 1985; Wolin and Walter 1988; for review, see Kozak 1991), at a site where an aminoacyl-tRNA is limiting (Scornik 1988), at sites of interaction of the nascent peptide with another protein (Kim et al. 1991), and perhaps also at other sites determined by nonsense or rare codons, frameshifting, ribosome hopping, or folding of the nascent peptide within the ribosomal tunnel or at the exit domain (Chen et al. 1984; Hoekema et al. 1987; Ross 1988; Theodorakis et al. 1988; Atkins et al. 1990; Ito et al. 1990; Picking et al. 1991). The *c-myc*-coding region determinant encodes

A

ACCAGATCCCGGAGTTGGAAAACAATGAAAAGGCCCCCAAGGTAGTTATC
 CTTAAAAAGCCACAGCATACATCCTGTCCGTCCAAGCAGAGGAGCAAAA
 GCTCATTTCTGAAGAGGACTTGTTCGCGAAACGACGAGAACAGTTGAAAC
 ACAAACCTGAACAGCTACGGAACTCTTGTGCG

B

Figure 9. Structure and possible function of the 182-nucleotide coding region determinant of *c-myc* mRNA stability and the ~75-kD binding protein. (A) Sequence. Purines comprise 58% of this sequence. The terminal GCG is the last codon before the terminator. (B) Model for possible links between translation, ribosome pausing, binding of the putative ~75-kD protective protein to the coding region determinant, and blocking of an endonuclease-sensitive site. The stacked circles indicate ribosomes translating the mRNA (see text for details).

part of the helix-loop-helix and all of the leucine zipper. It can potentially form several duplex segments, as determined both by computer modeling and by chemical and enzymatic digestion studies (J. Lee, pers. comm.). It also contains 58% purines, some clustered, similar to the destabilizing segment in the *c-fos* mRNA-coding region (Fig. 9A; Shyu et al. 1990). Thus, it seems reasonable to propose that a ribosome-free region could result from one of two processes: (1) ribosome stalling enhanced by binding of the ~75-kD protein; or (2) ribosome stalling occurring independent of the protein, followed by binding of the protein to the ribosome-free region. Experiments are in progress to investigate these alternatives and to determine whether the ~75-kD protein regulates translation as well as mRNA stability.

In either case, the existence of separate, independently regulated stability determinants in a single mRNA would permit cells to respond in different ways to changes in nutrient levels, growth factors, and differentiation agents. For example, a differentiation factor might influence the abundance of the ~75-kD protein described here, whereas a growth factor might affect a

different regulatory protein that interacts with the 3'-UTR. Recent experimental evidence supports this notion. A region of *c-myc* mRNA (encompassing the one described here) functions as an mRNA destabilizing element in a cell line capable of differentiating to form myotubes in culture (Wisdom and Lee 1990, 1991). Furthermore, evidence consistent with this notion and with alternate intracellular decay pathways was obtained by comparing *c-myc* mRNA decay rates in growing versus differentiating promyelocytic tissue culture cells (Swartwout and Kinniburgh 1989). In exponentially growing cells, deadenylation preceded destruction of the mRNA body, similar to the two-step pathway observed in reactions lacking competitor RNAs (Brewer and Ross 1988). In contrast, when the promyelocytic cells were induced to differentiate to myelocytes, *c-myc* mRNA disappeared without going through a deadenylation step, suggesting decay by a novel endonucleolytic pathway. Perhaps this pathway was induced by modifying *c-myc* mRNA-protein interactions, as suggested by our in vitro data. To test this possibility, the half-lives and decay pathways of modified *c-myc* mRNAs lacking either the coding or the

3'-UTR determinant can be compared during promyelocyte differentiation.

Materials and methods

In vitro mRNA decay reactions

The culturing of human erythroleukemia cells (K562) and the isolation of polysomes, which are the source of both endogenous messenger ribonucleoprotein (mRNP) and mRNA-degrading enzymes, were performed as described previously (Ross and Kobs 1986; Ross et al. 1987). Briefly, exponentially growing cells were harvested, washed in cold F12 medium, and lysed with a Potter-Elvehjem homogenizer in low salt buffer [1 mM potassium acetate, 1.5 mM magnesium acetate, 2 mM dithiothreitol (DTT), 10 mM Tris-HCl at pH 7.6]. After removal of nuclei by low-speed centrifugation, polysomes were pelleted at 130,000g through a cushion of 30% sucrose in low salt buffer, resuspended in low salt buffer, and frozen. RSW was prepared as described previously, except the washing buffer contained 10% glycerol and 1.0 M NaCl, rather than 0.5 M KAc (Ross et al. 1987).

Cell-free mRNA decay reactions containing 0.7 A_{260} units of polysomes (~25 μ g of RNA) were performed as described previously (Ross et al. 1987). Competitor RNAs (see below) were added, where noted, at 1 μ g per 25 μ g of polysomal RNA. After incubation at 37°C, total RNA was prepared by phenol extraction.

Preparation of competitor RNAs

Plasmid pSP6ClaRI or pSP6RICla (sense and antisense-oriented clones from the *Cla*I site in *c-myc* exon 3 to the *Eco*RI site in the 3'-flanking region) was linearized and used in SP6 RNA polymerase transcription reactions with synthetic cap analog, as described previously (see Fig. 1; Peltz et al. 1987). DNA fragments subcloned from the coding region of human β -globin cDNA and from pGEM vector DNA were similarly transcribed to generate nonspecific competitor RNAs. The DNA template for RNA *d* (Fig. 1) was synthesized by polymerase chain reaction (PCR) using a 5' single-stranded DNA primer containing SP6 promoter sequences plus *c-myc* cDNA bases 1705–1722 and a 3'-antisense primer from *c-myc* cDNA bases 1869–1886, the 1886 site being the third base of the last coding codon (amino acid 439). This DNA was either used directly in an SP6 transcription reaction or was subcloned into a vector lacking an SP6 promoter. In each experiment, vectors were cut at restriction enzyme sites such that the *c-myc* and nonspecific competitor RNAs were similar or identical in size.

Analysis of polysome-associated mRNAs

Northern blot analyses were performed as described by Church and Gilbert (1984) using Zeta-Probe membranes (Bio-Rad). The *c-myc* blotting probe indicated in Figure 1 was an *Ava*I fragment that does not hybridize to competitors *a* through *d*. Full-length cDNA probes were used for detecting *c-myc*, AP-4, γ -globin, and GAPDH mRNAs. All blotting probes were radiolabeled with 32 P by random priming (Feinberg and Vogelstein 1983). Equal RNA loading was determined in all experiments by stripping the blot and reprobing for a stable transcript such as γ -globin mRNA. RNase protection of *c-myc* mRNA, using a uniformly labeled 3' probe (Fig. 1), and S1 nuclease analyses of the 3' regions of histone and γ -globin mRNAs were performed

as described previously (Ross and Kobs 1986; Brewer and Ross 1988).

Preparation of radiolabeled RNAs and gel-shift analysis

Linearized templates (*c-myc* or β -globin cDNAs or pGEM vector DNA) were used in SP6 transcription reactions containing [α - 32 P]GTP to generate uncapped, radiolabeled RNAs. One nanogram of RNA was heated to 70°C for 5 min, chilled on ice, and incubated with 0.4 A_{260} units of K562 polysomes (or an equivalent amount of RSW protein eluted from polysomes in 1.0 M NaCl) in 10 μ l containing 2 mM DTT, 2 mM magnesium acetate, 0.4 mM spermine, 10% glycerol, 2.5 μ g/ μ l of tRNA, and 10 mM Tris-HCl (pH 7.5). The reaction was incubated at 30°C for 10 min and was then treated with 1 unit of RNase T1 at 30°C for 10 min. Heparin (50 μ g) was added to minimize nonspecific interactions. RNA-protein complexes were resolved from free RNA in a 6% nondenaturing polyacrylamide gel and visualized by autoradiography (Konarska and Sharp 1986). Where indicated, 4 A_{260} units of polysomes or an equivalent amount of RSW was pretreated with 10 μ g of trypsin for 30 min at 37°C; 10 μ g of soybean trypsin inhibitor was then added to inhibit further protease activity.

UV-cross-linking analysis

Mock mRNA decay reactions were carried out as described for the gel-shift analyses and were subsequently exposed for 5 min to 254 nm UV light at a distance of 3 cm (energy = 0.5 J) in a Stratagene Stratalinker apparatus, to allow covalent bond formation between protein and radiolabeled RNA (Bohjanen et al. 1991). Reactions were then treated with 8 μ g of RNase A for 60 min at 37°C, to digest non-cross-linked RNA. Radiolabeled proteins were analyzed by electrophoresis in a 10% SDS-polyacrylamide gel under reducing conditions and were visualized by autoradiography.

Construction of GlobMycGlob chimeric gene

A 249-bp DNA fragment corresponding to the carboxy-terminal 83 *c-myc* codons was prepared from *c-myc* cDNA by PCR. The 5' and 3' primers were 26-mer (5'-GCGAATTCCGAACACA-CAACGTCTTG) and 27-mer (5'-CTCGAATTCCGCACAA-GAGTTCCGTAG), respectively. Each primer contained *Eco*RI restriction sites, which permitted insertion of this fragment into the *Eco*RI site of a human β -globin gene subcloned into pBS.KS M13+ (Stratagene). The orientation and sequence of the resulting GlobMycGlob clone were verified by T7 sequencing (Pharmacia). This clone was then inserted into the *Hind*III and *Nsi*I sites of plasmid pB108, a eukaryotic expression vector containing the CMV immediate early promoter and 112 bp of CMV 5'-untranslated sequence (Ghazal and Nelson 1991) plus the *Escherichia coli* hygromycin phosphotransferase gene (Gritz and Davies 1983). The CMV-GlobMycGlob gene expresses a 1006-nucleotide mRNA containing, in a 5' $\rightarrow$ 3' order, 112 nucleotides of CMV exon 1, 13 nucleotides from a previously added *Hind*III linker, 419 nucleotides from the β -globin cap site to the *Eco*RI site (complete 5'-UTR plus codons for globin amino acids 1–121), 249 nucleotides from the terminal coding region of *c-myc* mRNA (amino acids 357–439), 6 nucleotides from the *Eco*RI linker, encoding Gln-Phe, and 207 nucleotides from the β -globin *Eco*RI site to the mRNA terminus (codons for globin amino acids 122–146 plus complete 3'-UTR). This mRNA should utilize the normal β -globin translation initiation and termination signals, and the *c-myc* segment should be translated as well (see Fig. 6A).

HeLa cell culture and transfection

Adherent HeLa cells were grown on 100-mm dishes in α -MEM plus 10% calf serum (GIBCO) supplemented with penicillin and streptomycin. Subconfluent cells ($\sim 5 \times 10^6$ per dish) were transfected with 20 μ g of CMV-GlobMycGlob DNA, using lipofectin reagent (GIBCO BRL). A pool of cells was then selected using 400 μ g/ml of hygromycin B (Boehringer Mannheim). Poly-some isolation and in vitro mRNA decay reactions were performed as described for the K562 cells.

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