c-myc mRNA Is Down-regulated during Myogenic Differentiation by Accelerated Decay That Depends on Translation of Regulatory Coding Elements*

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Murine C2C12 myoblasts induced to differentiate into multinucleated myotubes decrease their levels of c-myc mRNA 3–10-fold through posttranscriptional mechanisms that recognize regulatory elements contained in protein-coding sequences in exons 2 and 3 of the mRNA. To determine the mechanism by which these elements mediate c-myc mRNA down-regulation, we examined the regulation of mutant MYC and human β-globin-MYC fusion mRNAs. Regulation of mRNAs containing MYC exon 2 or 3 is abolished by insertion of an upstream termination codon indicating that regulatory function depends on their translation. Exploiting this translation dependence, we show that pharmacologic inhibition of translation with cycloheximide abolishes the down-regulation of regulated MYC and globin-MYC mRNAs and induces their levels in differentiating C2C12 cells. We exclude the possibility that this induction in mRNA levels results from cycloheximide effects on transcription or processing of parts of the RNA other than the regulatory elements, leading to the conclusion that cycloheximide induction results from mRNA stabilization. We show that the magnitude of cycloheximide induction can be used to estimate turnover rates of mRNAs whose decay is translation-dependent. By using cycloheximide inducibility to examine turnover rates of MYC and globin-MYC mRNAs, we show that the MYC exon 2 and exon 3 regulatory elements, but not MYC 3′-untranslated region or chloramphenicol acetyltransferase coding sequences, mediate accelerated mRNA decay in differentiating, but not undifferentiated, C2C12 cells. We show that these regulatory elements must be translated to confer accelerated mRNA decay and that increased turnover occurs in the cytoplasm and not in the nucleus. Finally, using cycloheximide induction to examine mRNA half-lives, we show that mRNA turnover is increased sufficiently by mechanisms targeting the exon 2 and 3 regulatory elements to account for the magnitude of c-myc mRNA down-regulation during differentiation. We conclude from these results that c-myc mRNA down-regulation during myogenic differentiation is due to translation-dependent mechanisms that target mRNAs containing myc exon 2 and 3 regulatory elements for accelerated decay.

mRNA turnover rates play an important role in determining levels of cellular gene expression, and regulated mRNA turnover has been shown to regulate expression of a number of genes. mRNA stability has been shown to be regulated through a variety of mechanisms that target specific RNA sequences or motifs, RNA secondary structural elements, or the encoded peptide. For example, many labile cytokine and proto-oncogene mRNAs (e.g. c-fos and granulocyte macrophage-colony-stimulating factor) contain AU-rich/AUUUA sequence elements in their 3′-untranslated regions (UTRs) that target them for rapid turnover and limit gene expression (1, 2), whereas α-globin mRNA has a C-rich element in its 3′-UTR that allows formation of a protein-mRNA complex that is thought to stabilize the mRNA and allow prolonged gene expression (3). A secondary structural element in the 3′-UTR of histone mRNA targets it for translation-dependent turnover that is coupled to the position of the cell in the cell cycle and DNA synthesis (4). Turnover of transferrin receptor mRNA is regulated by stem-loop structures in its 3′-UTR termed iron-responsive elements that are bound by iron regulatory proteins in an iron-poor environment resulting in mRNA stabilization. Conversely, in an iron-rich environment, the unprotected mRNA is rapidly degraded. Autoregulation of β-tubulin mRNA levels occurs through recognition of the first four amino acids of the nascent peptide by excess free β-tubulin subunits, and resulting dimerization targets the mRNA for accelerated turnover (5).

Regulated RNA stability plays a critical role in controlling expression of c-myc, a proto-oncogene encoding a transcription factor important in regulating cell proliferation and differentiation. The short half-life of c-myc mRNA (15–30 min) (6, 7) allows cells to rapidly alter c-myc expression either through transcriptional (8–12) or posttranscriptional (13–18) mechanisms. A decrease in c-myc mRNA expression is seen when cells are induced to differentiate (13–18), and a rapid increase is seen following mitogen exposure (19). We have been characterizing posttranscriptional mechanisms controlling c-myc mRNA levels in C2C12 murine myoblasts (20, 21), and we have demonstrated previously that sequences in the 3′-UTR determine the turnover rate and steady-state levels of c-myc mRNA in proliferating C2C12 cells. However, these sequences are dispensable for the posttranscriptional down-regulation of c-myc mRNA levels when C2C12 cells are induced to differentiate into multinucleated myotubes (17, 20, 21). Instead, coding elements in myc exons 2 and 3 target c-myc mRNA for down-regulation during differentiation. In studies presented here, we characterize the mechanism by which c-myc expression is post-

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† The abbreviations used are: UTR, untranslated region(s); DMEM, Dulbecco’s minimum essential medium; DM, differentiation media; MLV, murine leukemia virus; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; RT-PCR, reverse transcriptase-polymerase chain reaction.
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**A** mycDeletion Mutants

| Human c-myc | Myc (XN) | Myc (Δ41–178) | Myc (265–433) | Myc (TN) | Myc (Es2Term3) | Myc (Es32) |
|-------------|----------|---------------|---------------|----------|----------------|------------|
| X          | T        | P             | T             | N        | T              | N          |

**B** Globin-MyC Fusion Genes

| MLV β-globin | Gm434SVpA | Gm363SVpA | Gm463-263SVpA | Gm4037TermSVpA | Gm463-263TermSVpA |
|--------------|-----------|-----------|---------------|-----------------|-------------------|
|              | +         | +         | +             | +               | +                 |

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**Fig. 1.** Schematic diagram of recombinant genes used in these studies. Construction of these genes is outlined under “Experimental Procedures.” Structural features of these genes and the shading pattern of exonic sequences are depicted below the genes. An NcoI restriction site is introduced adjacent to exon 4 of c-myc coding sequences from codon 263 to the NsiI site in c-MYC exon 3) are fused in-frame to γ-globin; and in Gm40–262SVpA, MYC mRNA sequences from codon 40–262 (most exon 2 coding sequences and the first 10 codons in exon 3) are fused in-frame to globin. To create Gm263TermSVpA and Gm40–262TermSVpA, a termination codon was inserted into the linker fusing globin and MYC domains allowing globin sequences, but not MYC sequences, to be translated. βGM-263SVpA and βGM-40–262SVpA were created by introducing a C to T mutation by site-directed mutagenesis into β-globin codon 122 which destroys an EcoRI site but preserves the encoded amino acid (phenylalanine). In β-CAT, the MLV-β-globin backbone is fused to the last 181 codons (out of a total of 219) of the bacterial gene encoding chloramphenicol acetyltransferase (CAT) and uses the SVpA. Analysis of mRNA Levels in Stably Transfected Cells—RNA isolation for differentiation assays was described previously (21). To examine induction in mRNA levels after inhibition of translation, total cytoplasmic mRNA was isolated from duplicate culture plates of both undifferentiated and differentiating C2C12 cells, one plate untreated and the other exposed to cycloheximide (Sigma) at a concentration of 10 μg/ml for 3 h, unless otherwise stated. Differentiating cells were used 48 h after exposure to DM, and differentiation-induced DM mRNA down-regulation was considered to have occurred when c-myc mRNA levels decreased more than 3-fold compared with levels in undifferentiated cells. mRNA levels were determined by Northern analysis using the glyoxyl method (24). RNA was electroblotted to Hybond N (Amersham Pharmacia Biotech) and UV cross-linked. Hybridizations were carried out by modifications of the method of Church and Gilbert (25) using probes labeled by random priming. Human MYC mRNA were probed using a human c-MYC exon 1 probe (XhoI to PvuII fragment) or a human c-MYC exon 2 + 3 DNA probe from pSP65MYCIIA (23). β-Globin chimeric mRNAs were probed using a full-length human β-globin cDNA fragment from pSPβex (gift from Stephen Liebhaber). C2C12 c-myc mRNA was probed with a murine c-myc exon 1 probe (BamHI to NotI fragment) or a human c-myc exon 2 + 3 cDNA probe from pS65MYCIIA (23). RPL32 mRNA was probed with a full-length cDNA probe (26). Northern blots were analyzed on a Molecular Dynamics PhosphorImager (Sunnyvale, CA) using ImageQuant software, and the relative levels of MYC and globin-MYC fusion mRNAs during differentiation or after cycloheximide were determined by normalizing for RNA loading using the level of rpl32 mRNA. All experiments were performed at least twice, and representative results are displayed in the figures presented.

**Derivation of mRNA Half-lives by Analysis of Induction of mRNA Levels by Cycloheximide—**Induction in mRNA levels 3 h after inhibition of translation was used to deduce MYC and globin-MYC mRNA half-lives. Assuming first order kinetics of mRNA decay, the steady-state level of a given mRNA species, [mRNA]ss, is a function of its rate of synthesis, kss, and its half-life, t1/2, and can be calculated as shown in Equation 1.

\[ [\text{mRNA}]_{ss} = \frac{k_{ss} \times [\text{mRNA}]_{0}}{t_{1/2}} \]  

(Eq. 1)

If mRNA decay is completely inhibited but the rate of transcription continues at the same rate, the mRNA level at time t, [mRNA]t, will be...
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Under these conditions, the fold induction of a given mRNA species, [mRNA]_{end} - [mRNA]_{sta}, is a function of the length of time synthesis is allowed to continue and the mRNA half-life prior to stabilization as shown in Equation 3.

\[ \text{fold induction} = \frac{[\text{mRNA}]_{end} - [\text{mRNA}]_{sta}}{[\text{mRNA}]_{sta}} + (t_{1/2})/t_{1/2} \] (Eq. 3)

Therefore, the mRNA half-life can be calculated under these conditions as shown in Equation 4.

\[ t_{1/2} = (t_{1/2})/2 \] (fold induction 1) (Eq. 4)

Results

Murine C2C12 myoblasts are induced to differentiate into multinucleated myotubes by mitogen deprivation. c-myc expression is down-regulated early during differentiation, prior to the up-regulation of muscle-specific genes (e.g. creatine kinase) (17). This occurs through posttranscriptional mechanisms demonstrated by nuclear run-on assays which showed that the rate of c-myc transcription does not change significantly during differentiation, whereas c-myc mRNA levels decrease 3–10-fold (17). We previously demonstrated that two protein coding elements, one in exon 2 and the other in exon 3, are necessary for targeting myc mRNA for down-regulation, whereas 5' and 3' UTR sequences and introns are dispensable.

Translation of Myc Exon 3 Is Required for Myc mRNA Down-regulation during Differentiation—The presence of regulatory determinants within the protein coding region of c-myc mRNA raises the possibility that their recognition may be coupled to translation. To determine whether translation of the myc exon 3 regulatory element is required for down-regulation, C2C12 cells were stably transfected with MYC(T/N), a MYC cDNA construct from which most 5’- and 3’-UTR sequences were deleted, or with MYC(Ex2Term3), a construct identical to MYC(T/N) except for the insertion of an in-frame nonsense mutation at codon 262 that prevents translation of about 95% of the 5’ regulatory element. Cytoplasmic RNA was isolated on serial days from subconfluent cells, confluent cells, and confluent cells induced to differentiate. Northern analysis demonstrated that MYC(Ex2Term3) mRNA was not down-regulated and even increased over 3-fold, whereas levels of endogenous c-myc mRNA in the same cells were down-regulated 6.7-fold (Fig. 2B). This showed that translation of the myc exon 3 regulatory element is required for myc mRNA down-regulation.

The Order of Myc Exon 2 and 3 Regulatory Sequences Affects
My c-myc mRNA Down-regulation—The translation dependence of exon 3 regulatory function made it impossible to determine whether function of the myc exon 2 regulatory element is translation-dependent in its normal context because insertion of a stop codon upstream of exon 2 would prevent translation of both elements. However, if the relative positions of the exon 2 and exon 3 elements are unimportant for myc regulation, the translation dependence of exon 3 regulatory function could be tested in a mutant mRNA in which the positions of the exon 2 and exon 3 coding elements are reversed. To explore this possibility, C2C12 cells were stably transfected with MYC(Ex3/2) in which the exon 3 regulatory element was placed 5' to the exon 2 regulatory element and maintains the proper reading frame of both. Northern analysis showed that MYC(Ex3/2) mRNA was not down-regulated during differentiation (Fig. 2C) demonstrating that the position of the exon 2 and 3 regulatory elements affects their function, precluding testing of the translation dependence of exon 3 regulatory function in the myc mRNA context.

Myc Regulatory Elements Must Be Translated to Confer Down-regulation onto Globin mRNA—To determine the importance of translation for exon 2 function, we examined regulation of chimeric mRNAs in which MYC sequences were fused in-frame to human β-globin mRNA. We previously showed that human β-globin mRNA was down-regulated during differentiation when fused to either the exon 2 or 3 regulatory element but not when fused to MYC 3'-UTR sequences or to CAT or rpL32 coding sequences (Ref. 21 and see Fig. 4). These globin-MYC chimeric genes allowed us to test the translation dependence of function of each of the myc mRNA regulatory domains independent of the other element. To that end, C2C12 cells were stably transfected with βGm(40–262)/TermSVpA and βGm263TermSVpA, constructs which contain a stop codon 5' to the MYC exon 2 or 3 regulatory element, respectively (ribosomes translate globin codons but not MYC codons). When C2C12 transfectants were induced to differentiate, levels of βGm(40–262)/TermSVpA mRNA decreased only 1.3-fold and levels of βGm263TermSVpA mRNA were essentially unchanged, whereas levels of endogenous c-myc mRNA decreased 4.8- and over 10-fold in these respective stable transfectants (Fig. 3, A and B). Thus, the regulatory elements in myc exons 2 and 3 must be translated to confer down-regulation on globin mRNA.

Cycloheximide Studies Reveal That Myc Exons 2 and 3 Contain Translation-dependent Instability Determinants—Whereas coding sequences in myc exons 2 and 3 serve as translation-dependent, conditional mRNA regulatory elements, the mechanism by which they down-regulate mRNA is unclear. If the regulatory elements mediate down-regulation by accelerating mRNA decay in differentiating C2C12 cells, inhibiting their function should result in stabilization and increased levels of their mRNAs. Since their function is translation-dependent, we determined whether function of the MYC regulatory elements mediate down-regulation by accelerating mRNA decay in differentiating C2C12 cells stably transfected with βGm(40–262)/TermSVpA, βGm263SVpA, βGm434MYCpA, or βG-CAT. Cytoplasmic RNA was isolated from preconfluent cells (lanes 1 and 2) and cells induced to differentiate for 36 h in differentiation media (lanes 3 and 4) that were untreated (+) or exposed to cycloheximide (CHX) for 3 h (+). Autoradiographs of Northern blots display mRNA levels in cells transfected with βGm(40–262)/TermSVpA (A), βGm263SVpA (B), βGm434MYCpA (C), and βG-CAT (D). mRNA from the transfected gene was detected using a full-length human β-globin cDNA probe, pSPβPc; endogenous murine c-myc mRNA was detected using a probe for the CAT coding region (B), or with a probe for the CAT coding region (D); the endogenous murine c-myc mRNA was detected using a probe for the Hu-MYC exon 2 + 3 coding region, and rpL32 mRNA was detected using a full-length cDNA probe (26). RNA from untransfected C2C12 cells (lane 5) demonstrates probe specificity for transgene mRNA. The fold induction in transgene mRNA levels after cycloheximide when normalized for RNA loading using the level of rpL32 mRNA is displayed. Fold induction (c-myc mRNA level in cycloheximide-treated cells + c-myc mRNA level in untreated cells) − (rpL32 mRNA level in cycloheximide-treated cells + rpL32 mRNA level in untreated cells).

Cycloheximide-induced decay of chimeric mRNAs was examined in C2C12 cells stably transfected with βGm(40–262)/TermSVpA, βGm263SVpA, βGm434MYCpA, or βG-CAT. Cytoplasmic RNA was isolated from preconfluent cells (lanes 1 and 2) and cells induced to differentiate for 36 h in differentiation media (lanes 3 and 4) that were untreated (+) or exposed to cycloheximide (CHX) for 3 h (+). Autoradiographs of Northern blots display mRNA levels in cells transfected with βGm(40–262)/TermSVpA (A), βGm263SVpA (B), βGm434MYCpA (C), and βG-CAT (D). mRNA from the transfected gene was detected using a full-length human β-globin cDNA probe, pSPβPc; endogenous murine c-myc mRNA was detected using a probe for the CAT coding region (B), or with a probe for the CAT coding region (D); the endogenous murine c-myc mRNA was detected using a probe for the CAT coding region (D); and cells induced to differentiate for 36 h in differentiation media (lanes 3 and 4) that were untreated (+) or exposed to cycloheximide (CHX) for 3 h (+). Autoradiographs of Northern blots display mRNA levels in cells transfected with βGm(40–262)/TermSVpA (A), βGm263SVpA (B), βGm434MYCpA (C), and βG-CAT (D). mRNA from the transfected gene was detected using a full-length human β-globin cDNA probe, pSPβPc; endogenous murine c-myc mRNA was detected using a probe for the CAT coding region (B), or with a probe for the CAT coding region (D); and cells induced to differentiate for 36 h in differentiation media (lanes 3 and 4) that were untreated (+) or exposed to cycloheximide (CHX) for 3 h (+). Autoradiographs of Northern blots display mRNA levels in cells transfected with βGm(40–262)/TermSVpA (A), βGm263SVpA (B), βGm434MYCpA (C), and βG-CAT (D). mRNA from the transfected gene was detected using a full-length human β-globin cDNA probe, pSPβPc; endogenous murine c-myc mRNA was detected using a probe for the CAT coding region (B), or with a probe for the CAT coding region (D); and cells induced to differentiate for 36 h in differentiation media (lanes 3 and 4) that were untreated (+) or exposed to cycloheximide (CHX) for 3 h (+). 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Autoradiographs of Northern blots display mRNA levels in cells transfected with βGm(40–262)/TermSVpA (A), βGm263SVpA (B), βGm434MYCpA (C), and βG-CAT (D). mRNA from the transfected gene was detected using a full-length human β-globin cDNA probe, pSPβPc; endogenous murine c-myc mRNA was detected using a probe for the CAT coding region (B), or with a probe for the CAT coding region (D); and cells induced to differentiate for 36 h in differentiation media (lanes 3 and 4) that were untreated (+) or exposed to cycloheximide (CHX) for 3 h (+). Autoradiographs of Northern blots display mRNA levels in cells transfected with βGm(40–262)/TermSVpA (A), βGm263SVpA (B), βGm434MYCpA (C), and βG-CAT (D). mRNA from the transfected gene was detected using a full-length human β-globin cDNA probe, pSPβPc; endogenous murine c-myc mRNA was detected using a probe for the CAT coding region (B), or with a probe for the CAT coding region (D); and cells induced to differentiate for 36 h in differentiation media (lanes 3 and 4) that were untreated (+) or exposed to cycloheximide (CHX) for 3 h (+).
This contrasted with a 7- and 17-fold induction in the endogenous c-myc mRNA, respectively, demonstrating that the insignificant induction in βGM(40–262)SVpA and βGM263-SVpA mRNA levels in undifferentiated cells was due not to lack of cycloheximide effect (in all our cycloheximide studies, induction of endogenous c-myc mRNA levels was used as a positive control for cycloheximide effect). These results indicate that the destabilization function of the myc exon 2 and exon 3 regulatory elements requires differentiation.

While the foregoing results support a translation-dependent destabilization function of myc exon 2 and 3 regulatory elements, cycloheximide could also be affecting transcription from the MLV LTR or regulation from non-myc portions of the mRNAs. To exclude these possibilities, the effect of cycloheximide was examined in C2C12 cells stably transfected with (i) βG-CAT in which CAT sequences are fused in-frame to globin in place of myc sequences, or (ii) βGm434MYCpA which contains the last six MYC 3′-UTR. Levels of mRNAs encoded by these genes were induced no more than 2-fold by cycloheximide in either preconfluent or differentiating C2C12 cells (Fig. 4, C and D). Cycloheximide inducibility depends on mRNA instability being translation-dependent, but the magnitude of induction depends on the turnover rate of the mRNA prior to stabilization, i.e. a given increase in the amount of a stable mRNA is fractionally smaller and more difficult to appreciate than the same increase in the amount of an unstable mRNA. βGm434MYCpA mRNA has a decay rate comparable to βGm263SVpA mRNA under growth conditions (20) and should be comparably induced if cycloheximide affected its metabolism, e.g. through increased transcription from the MLV LTR. These results, therefore, exclude the possibility that cycloheximide induction of βGm40–262SVpA and βGm263SVpA mRNAs resulted from increased MLV LTR transcription or from cycloheximide effects on globin or SV40 polyadenylation sequences during differentiation. Additionally, they show that MYC sequences that do not confer down-regulation during C2C12 differentiation (i.e. the 3′-UTR) do not confer cycloheximide inducibility. Thus, sequences from either MYC exons 2 or 3 specifically confer cycloheximide inducibility on β-globin and function as translation-dependent mRNA instability determinants during C2C12 differentiation. If transcription and mRNA processing rates are unchanged by cycloheximide, the 3–6-fold difference in cycloheximide inducibility between undifferentiated and differentiating cells suggests that the decay rates of these mRNAs are accelerated 3–6-fold in differentiating cells (see “Discussion”).

**Coding Sequences in Both Exons 2 and 3 and Their Translation Are Necessary for Cycloheximide-induction of myc mRNA during Differentiation**—If accelerated myc mRNA decay accounts for its down-regulation during C2C12 differentiation, mutations that disrupt myc mRNA down-regulation should prevent its accelerated decay. To test this hypothesis, we examined the effect of cycloheximide on the following: (a) MYC(Δ41–178) mRNA, a mutant human MYC mRNA from which most of exon 2 was deleted; (b) MYC(Δ265–433) mRNA, a MYC mutant from which most exon 3 coding sequences were deleted; (c) MYC(ΔN) mRNA, a MYC mutant from which most 3′-UTR sequences were deleted; or (d) MYC(T/N) mRNA, a MYC mutant from which most 5′- and 3′-UTR sequences were deleted. Previously, we showed that MYC(Δ41–178) and MYC(Δ265–433) mRNAs are not down-regulated and that MYC(ΔN) and MYC(T/N) mRNAs are down-regulated during differentiation (21). MYC(Δ41–178) and MYC(Δ265–433) mRNAs were very modestly induced by cycloheximide treatment in preconfluent cells but were not induced in differentiating C2C12 cells, whereas levels of endogenous c-myc mRNA were induced (Fig. 5, A and B). In contrast, MYC(ΔN) and MYC(T/N) mRNAs, which were poorly induced by cycloheximide in preconfluent cells, were induced 3.2- and 5.5-fold, respectively, in differentiating cells (Fig. 5, C and D). Thus, mutant MYC mRNAs that are down-regulated during C2C12 differentiation are cycloheximide-inducible, whereas those that are not down-regulated during differentiation are not cycloheximide-inducible. These results demonstrate that coding sequences in both myc exons 2 and 3 are necessary for cycloheximide induction of myc mRNA and support the hypothesis that they, but not 5′ or 3′-UTR sequences, are necessary for translation-dependent accelerated c-myc mRNA decay during C2C12 differentiation.

If accelerated mRNA decay during differentiation is dependent on translatability of the myc exon 2 and 3 regulatory elements, we would predict that cycloheximide inducibility should also depend on the translatability of these elements. As expected if myc exon 3 sequences must be translatable to confer cycloheximide inducibility, levels of MYC(Ex2Term3) mRNA were not induced by cycloheximide in either preconfluent or differentiating cells (Fig. 6A). Since the translation dependence of exon 2 sequences cannot be examined in myc mRNA without perturbing translation of exon 3 sequences, we examined the effect of their translatability on cycloheximide inducibility using βGm40–262/TermSVpA. Levels of βGm(40–262)/Term-SVpA mRNA were not induced after cycloheximide treatment in either preconfluent or differentiating cells (Fig. 6B) showing that...
Fig. 6. MYC exon 2 and 3 regulatory elements must be translatable to confer regulated cycloheximide inducibility (CHX Ind) during myoblast differentiation. Induction in levels of MYC or globin-MYC fusion mRNAs was examined in C2C12 cells stably transfected with MYC(Ex2Term3) or βGM(40–262)TermSVpA. Cytoplasmic RNA was isolated from confluent cells (lanes 1 and 2) and cells induced to differentiate for 36 h in differentiation media (lanes 3 and 4) that were untreated (−) or exposed to cycloheximide for 3 h (+). Autoradiographs of Northern blots display mRNA levels in cells transfected with MYC(Ex2Term3) (A) and βGM(40–262)TermSVpA (B). mRNA from the transfected gene was detected with a probe for the exon 2 or 3 coding region of human (Hu) c-MYC (A) or a full-length human β-globin cDNA probe (B), the endogenous murine (Mo) c-myc mRNA was detected using a probe for the human myc exon 2 + 3 coding region, and rpl32 mRNA was detected using a full-length cDNA probe (26). RNA from untransfected C2C12 cells (lane 5) demonstrates probe specificity for transgene mRNA. The fold induction in transgene mRNA levels after cycloheximide when normalized for RNA loading using the level of rpl32 mRNA is displayed. Fold induction = (c-myc mRNA level in cycloheximide-treated cells + c-myc mRNA level in untreated cells) + (rpl32 mRNA level in cycloheximide-treated cells + rpl32 mRNA level in untreated cells).

Coding elements in both myc exons 2 and 3 must be translatable to confer regulated cycloheximide inducibility, i.e. accelerated mRNA decay during C2C12 differentiation.

Induction in Levels of c-myc mRNA by Cycloheximide Increases when C2C12 Myoblasts Differentiate—Measurements of c-myc mRNA decay after actinomycin D show only a modest increase in its turnover rate in differentiating cells compared with undifferentiated cells (19). It is likely that these results overestimate the half-life of c-myc mRNA in differentiating cells because actinomycin D interferes with the destabilizing function of myc exon 3 coding sequences (19), and its effects on myc exon 2 regulatory function are unknown. To determine whether c-myc mRNA turnover is accelerated during differentiation, we examined cycloheximide induction of c-myc mRNA levels in undifferentiated and differentiating C2C12 myoblasts. After 3 h of cycloheximide treatment, levels of c-myc mRNA were induced 11–14-fold in preconfluent and confluent cells, respectively (Fig. 7, lanes 1–4), whereas levels were induced 63-fold in differentiating cells (Fig. 7, lanes 5 and 6). This greater level of induction resulted more from the lower c-myc mRNA levels in differentiating cells not exposed to cycloheximide (Fig. 7, compare lanes 1, 3, and 5) than from higher levels of c-myc mRNA after cycloheximide (Fig. 7, compare lanes 2, 4, and 6). This 4–6-fold increase in cycloheximide inducibility at 3 h suggests that the turnover of c-myc mRNA is 4–6-fold faster in differentiating C2C12 cells which is consistent with the magnitude of down-regulation seen during differentiation and with estimates of the degree of destabilization imposed on globin mRNAs by the myc regulatory elements.

Accelerated mRNA Decay during Differentiation Affects Cytoplasmic But Not Nuclear mRNA Levels—Previous studies demonstrated that myc exon 2- or 3-mediated mRNA down-regulation during C2C12 differentiation affects cytoplasmic but not spliced nuclear mRNA levels (21). If accelerated mRNA decay is responsible for down-regulation, one would predict that it too should be a cytoplasmic event. To examine whether cytoplasmic RNA turnover is accelerated during differentiation, we examined the effect of cycloheximide on nuclear and cytoplasmic mRNA levels using an RT-PCR-based assay for comparing the relative abundance of two globin-MYC fusion mRNAs (20). To examine the effect of myc exon 2 coding sequences on cytoplasmic mRNA turnover, C2C12 cells were stably co-transfected with βGM(40–262)SVpARI, which encodes an mRNA that is induced by cycloheximide in differentiating cells, and βGM434SVpA, which encodes a noninducible mRNA. βGM(40–262)SVpARI is identical to βGM(40–262)SVpA except for a silent C to T mutation introduced at globin codon 122 that destroys an EcoRI site but does not affect mRNA processing or stability (20). By RT-PCR+1 analysis, the comparative level of spliced βGM434SVpA:βGM(40–262)SVpARI mRNAs in a nuclear RNA preparation from undifferentiated C2C12 cells was 1:1 (Fig. 8, lane 5). Their comparative levels did not change when cells were induced to differentiate (Fig. 8, lane 7) or when undifferentiated or differentiating cells were exposed to cycloheximide (Fig. 8, lanes 6 and 8), demonstrating that nuclear mRNA levels are not affected by exon 2 sequences during differentiation or after cytoplasmic decay. The relative abundance of these mRNAs in a cytoplasmic RNA preparation from undifferentiated cells was 1.3:1 (Fig. 8, lane 1) but changed to 3.9:1 when cells were induced to differentiate (Fig. 8, lane 3), demonstrating that βGM(40–262)SVpARI mRNA was down-regulated 3-fold compared with βGM434SVpA mRNA. In undifferentiated cells exposed to cycloheximide, the comparative cytoplasmic mRNA ratio was 1.1:1, an insignificant change from the ratio in untreated cells (Fig. 8, compare lanes 1 and 2). However, in differentiating cells exposed to cycloheximide, the comparative mRNA ratio changed from the 3.9:1 ratio seen in untreated cells to 1.1:1, a 3.5-fold relative increase in cytoplasmic levels of βGM(40–262)SVpARI mRNA compared with βGM434SVpA mRNA (Fig. 8, compare lanes 3 and 4). These results are consistent with previous results demonstrating that fusion of coding elements from MYC exon 2 confers down-regulation on a globin-MYC fusion mRNA, and they validate results shown here demonstrating that exon 2 confers cycloheximide inducibility under conditions of differentiation. Furthermore, they demonstrate that cycloheximide affects cytoplasmic but not nuclear mRNA levels, suggesting that cytoplasmic but not nuclear turnover of myc mRNA is accelerated during differentiation. Analyses of βGM263SVpARI demonstrated that MYC exon 3 affected the cycloheximide inducibility of cytoplasmic, but not nuclear, mRNA under conditions of differentiation suggesting that its regulatory element also affects cytoplasmic mRNA turnover during differentiation (data not shown).
nuclear, mRNA levels. C2C12 cells were stably co-transfected with βGm(40–262)SVpA. Cytoplasmic (C) and nuclear (N) RNA were extracted from preconfluent cells (P) and cells induced to differentiate for 36 h in differentiation media (D) that were untreated or exposed to cycloheximide for 3 h. Comparative levels of mRNAs from the β-globin-MYC genes were determined by RT-PCR, and the products were resolved on a 6% denaturing polyacrylamide gel. Autoradiographs display the following RT-PCR products, B (i.e., not reverse-transcribed) are shown in lanes 19–22, EcoRI and RT-PCR cut (Ct) and uncut (U) products are labeled. Treated and untreated are designated as (+) and (−), respectively.

**DISCUSSION**

Regulation of expression of the c-myc proto-oncogene occurs through posttranscriptional mechanisms in many cell lines (13–18), and its down-regulation is thought to be a critical determinant in cell differentiation (15, 16, 28–32). We previously demonstrated that coding elements in the myc exons 2 and 3 are necessary for myc mRNA down-regulation during differentiation of C2C12 myoblasts and sufficient to confer down-regulation on globin mRNA (21). Studies presented here examine the mechanism by which this occurs. The results show that function of these regulatory elements depends on their translation. Because function of the myc regulatory elements is dependent on their own translation, we reasoned that a pharmacologic inhibitor of translation, like cycloheximide, should also block function of the translation-dependent regulatory elements, resulting in increased mRNA levels in differentiating cells. This was confirmed when levels of globin-MYC fusion mRNAs containing coding sequences from either MYC exon 2 or 3 were induced by cycloheximide in differentiating C2C12 cells but not in undifferentiated myoblasts. Cycloheximide inducibility was not seen in globin fusion mRNAs containing MYC 3′-UTR or CAT coding sequences and, therefore, could not have resulted from cycloheximide effects on transcription, globin mRNA metabolism, or SV40 polyadenylation function. The importance of myc exon 2 and 3 regulatory sequences in conferring cycloheximide inducibility in differentiating C2C12 cells was confirmed by demonstration that deletion of exon 2 or 3 regulatory sequences from MYC mRNA abolished inducibility, whereas inducibility was unaffected in MYC mRNAs in which 5′- and/or 3′-UTR sequences were deleted. Thus, using two independent approaches, the function of the elements in myc exons 2 and 3 that mediate mRNA down-regulation during differentiation was shown to be dependent on translation. Although translation in cis is clearly necessary for down-regulation, our results do not rule out the possibility that short-lived trans-acting factors are also involved in the regulation of myc mRNA.

The translation dependence of exon 2 in mediating down-regulation during C2C12 differentiation contrasts with the translation independence of another regulatory function ascribed to exon 2. Morello and co-workers (33) have suggested that myc exons 2 and 3 contain independent elements that posttranscriptionally modulate c-myc mRNA levels in transgenic mice based on tissue-specific expression levels and mRNA inducibility in liver regeneration and after inhibition of protein synthesis with cycloheximide. In their model, regulation conferred by exon 2 sequences was independent of its own translation (34). A different mechanism targeting exon 2 sequences for regulation is suggested by these studies which is difficult to reconcile with that suggested by our studies except that there are obvious differences in experimental systems. Thus, exon 2 sequences may play multiple roles in posttranscriptionally regulating myc mRNA levels, one under conditions of cell differentiation and another in hepatic regeneration and determining tissue-specific mRNA levels.

The mechanism by which c-myc mRNA is down-regulated during C2C12 differentiation involves accelerated mRNA decay as demonstrated by analyses of cycloheximide inducibility of MYC and globin-MYC fusion mRNAs. Treatment of cells with cycloheximide or other protein synthesis inhibitors has long been known to stabilize mRNAs encoded by early response genes resulting in induction in their levels (35–37). It has been presumed that stabilization of these mRNAs results from inhibition of translation, thus implying that decay of these mRNAs is coupled to translation, either directly or indirectly by the requirement of a short-lived trans-acting factor involved in mRNA decay. However, no studies have directly examined the mechanism by which these agents stabilize mRNAs, and translation inhibitors can have pleiotropic effects on cell metabolism (35–38). Studies here show that increased transcription does not account for mRNA cycloheximide inducibility (see above and Ref. 19); therefore, the increase in levels of mRNAs containing myc exon 2 and 3 sequences after cycloheximide is best explained by stabilization of previously unstable mRNAs. Kinetic considerations indicate that the rapidity of and fold increase in the level of an mRNA following its stabilization are a
function of its turnover rate prior to stabilization (see “Experimental Procedures”). This suggests that mRNAs containing myc exon 2 or 3 sequences are very rapidly turned over in a translation-dependent manner in differentiating C2C12 cells. Coupled with the observation that those mRNAs containing MYC exon 2 or 3 sequences that are cycloheximide-inducible are also down-regulated during C2C12 differentiation, it is reasonable to think of these regulatory elements as conditional (i.e. differentiation-associated), translation-dependent, mRNA instability determinants. Analyses of nuclear and cytoplasmic mRNA levels demonstrated these elements mediate accelerated mRNA decay in the cytoplasm.

The 3–10-fold decrease in c-myc mRNA levels observed during C2C12 differentiation would predict a 3–10-fold increase in the rate of its decay if accelerated mRNA turnover is entirely responsible for its down-regulation. The difference in cycloheximide inducibility of MYC and globin-MYC mRNAs seen in undifferentiated cells compared with differentiating cells strongly suggests that accelerated mRNA decay does account for the 3–10-fold myc mRNA down-regulation. If one assumes no change in transcription rates and complete mRNA stabilization after cycloheximide, the magnitude of increase in mRNA levels over time can be used to calculate mRNA half-lives (see “Experimental Procedures”). Although these assumptions demand caution in applying this calculation, we believe it likely that all of the constructs tested would be affected equally by any changes in transcription (since they are all transcribed from the same promoter/enhancer elements), and there is little reason to think that cycloheximide would stabilize their mRNAs to differing extents. Therefore, the magnitude of change in cycloheximide inducibility seen with different stages of cell differentiation should accurately reflect the magnitude of change in mRNA half-life. The 3–6-fold greater cycloheximide inducibility conferred by MYC exon 2 and 3 regulatory elements in differentiating cells compared with undifferentiated cells suggests that they confer approximately a 3–6-fold increase in mRNA turnover rates, and thus account for the magnitude of mRNA down-regulation. Confirming this suggestion, the magnitude of cycloheximide inducibility of c-myc mRNA was found to increase 4–6-fold when undifferentiated cells were compared with differentiating cells.

The suggestion that accelerated mRNA decay mediates c-myc mRNA down-regulation contrasts with studies of the decay rate of c-myc mRNA using actinomycin D. Results of these studies suggest that myc mRNA decay is only modestly accelerated in differentiating C2C12 cells and not to an extent that would explain the 3–10-fold decrease in steady-state mRNA levels (17). Actinomycin D studies and analyses of cycloheximide inducibility of c-myc mRNA predict similar half-lives for c-myc mRNA (approximately 15–30 min) in undifferentiated C2C12 cells. However, they yield different results in differentiating C2C12 cells with actinomycin D studies predicting a longer half-life of c-myc mRNA than do cycloheximide studies. The myc mRNA half-life predicted by actinomycin D studies in differentiating cells is likely artificiially long since actinomycin D interferes with the destabilizing function of myc exon 3 coding sequences (19) which is necessary for myc mRNA down-regulation, and its effects on myc exon 2 regulatory function are unknown. Studies presented here analyzing induction in mRNA levels after cycloheximide are unlikely to yield artificiual results because they reflect mRNA turnover rates prior to the addition of pharmacologic agents, and the mechanism of stabilization will not affect the kinetics of induction.

The mechanism by which exon 2 and 3 coding elements target c-myc mRNA for translation-dependent accelerated turnover during differentiation remains to be elucidated. A number of other mRNAs have been shown to be targeted for decay by protein coding region instability determinants. Like c-myc mRNA, c-fos (2), and β-interferon (39) mRNAs have been shown to contain independent instability determinants in both their protein coding domains and their 3′-UTRs. It remains to be determined whether the c-fos and β-interferon elements are simply redundant destabilizing elements or whether, like the c-myc coding region and 3′-UTR elements, they destabilize the mRNA under different conditions. Yeast MATα1 (40) and mammalian β-tubulin (5) mRNAs have also been shown to contain instability elements in their coding sequences. Of these various coding region instability determinants, the mechanism of recognition has been determined only for β-tubulin mRNA in which auto-regulation of β-tubulin mRNA levels depends on translation of its first four codons. Excess free β-tubulin monomers target β-tubulin mRNA for accelerated turnover by recognition of the encoded amino acids rather than the RNA sequence or structure (5). Our results have not excluded primary or secondary structure of the myc regulatory elements or the amino acid sequence encoded as the feature targeting c-myc mRNA for down-regulation. The stability of these and many labile mRNAs has been shown to be coupled to translation (for review, see Ref. 41). With mRNAs other than β-tubulin mRNA, the mechanism coupling translation to mRNA decay is unclear. Several models have been suggested including the following: (i) association of nucleases or other proteins involved in mRNA decay with the translation machinery; (ii) disruption by transiting ribosomes of RNA secondary structural elements important in RNA stability; (iii) dislocation of proteins involved in RNA stability by transiting ribosomes; or (iv) localization of the RNA to the subcellular region involved in RNA decay by the translation machinery (41). Our results do not exclude any of these possibilities as the mechanism by which translation targets c-myc mRNA for accelerated decay during differentiation.

In conclusion, we demonstrated that function of the exon 2 and exon 3 elements that mediate posttranscriptional down-regulation of c-myc mRNA during C2C12 differentiation is dependent on their translation. We have demonstrated that these elements mediate down-regulation through accelerated turnover of cytoplasmic, but not nuclear, mRNA. Furthermore, the translation-dependent destabilizing function of these elements is conditional, destabilizing the mRNA in differentiating C2C12 cells but not in undifferentiated myoblasts. Thus, c-myc mRNA is regulated posttranscriptionally by a variety of mechanisms. Under normal growth conditions, c-myc mRNA is maintained at low steady-state levels by translation-independent instability elements within the 3′-UTR (20). When C2C12 cells differentiate into multinucleated myotubes, c-myc mRNA is down-regulated through a translation-dependent mechanism targeting elements in the coding regions of exons 2 and 3 that function to destabilize the mRNA during differentiation but not in undifferentiated cells. These two mRNA regulatory pathways do not appear to interact and seem to function independently of each other. Thus, deletion of coding sequences in exons 2 and 3 does not affect steady-state myc mRNA levels under growth conditions, and deletion of myc 3′-UTR sequences does not affect down-regulation of c-myc mRNA during C2C12 differentiation. Their effects are superimposable, however, so that 3′-UTR sequences contribute to low steady-state myc mRNA levels during differentiation even though they do not confer regulation. Moreover, we have not ruled out the possibility that myc 3′-UTR-containing mRNAs reach lower steady-state levels in differentiating C2C12 cells than mRNAs without myc 3′-UTR sequences since their steady-state levels are lower prior to differentiation. Future studies will attempt to localize and define the elements in exons 2 and 3 targeting myc mRNA.
for down-regulation and identify the trans-acting factors that mediate its down-regulation.

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REFERENCES
1. Shaw, G., and Kamen, R. (1986) Cell 46, 659–667
2. Shyu, A.-B., Greenberg, M. E., and Belasco, J. G. (1989) Genes Dev. 3, 60–72
3. Wang, X., Kiledjian, M., Weiss, I. M., and Liebhaber, S. A. (1995) Mol. Cell. Biol. 15, 1769–1777
4. Graves, R. A., Pandey, N. B., Chodchoy, N., and Marzluff, W. F. (1987) Cell 48, 615–626
5. Zeng, T. J., Machlin, P. S., and Cleveland, D. W. (1988) Nature 335, 580–585
6. Dani, C., Blanchard, J. M., Piechaczyk, M., El Saboury, S., Marty, L., and Jeanteur, P. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 7046–7050
7. Jones, T. R., and Cole, M. D. (1987) Mol. Cell. Biol. 7, 4513–4521
8. Bentley, D. L., and Groudine, M. (1986) Nature 321, 702–706
9. Nepveu, A., and Marcu, K. B. (1986) EMBO J. 5, 2859–2865
10. Dean, M., Levine, R. A., and Campisi, J. (1986) Mol. Cell. Biol. 6, 518–524
11. Donzén, L. E., and Pitot, H. C. (1985) Cancer Res. 45, 847–850
12. Dean, M., Levine, R. A., and Campisi, J. (1986) Mol. Cell. Biol. 6, 518–524
13. Dean, M., Levine, R. A., and Campisi, J. (1986) Mol. Cell. Biol. 6, 518–524
14. Dean, M., Levine, R. A., and Campisi, J. (1986) Mol. Cell. Biol. 6, 518–524
15. Dean, M., Levine, R. A., and Campisi, J. (1986) Mol. Cell. Biol. 6, 518–524
16. Dean, M., Levine, R. A., and Campisi, J. (1986) Mol. Cell. Biol. 6, 518–524
17. Dean, M., Levine, R. A., and Campisi, J. (1986) Mol. Cell. Biol. 6, 518–524
18. Dean, M., Levine, R. A., and Campisi, J. (1986) Mol. Cell. Biol. 6, 518–524
19. Dean, M., Levine, R. A., and Campisi, J. (1986) Mol. Cell. Biol. 6, 518–524
20. Dean, M., Levine, R. A., and Campisi, J. (1986) Mol. Cell. Biol. 6, 518–524
21. Yeilding, N. M., and Lee, W. M. (1997) Mol. Cell. Biol. 17, 2698–2707
22. Blau, H. M., Pavlath, G. K., Hardeman, E. C., Chiu, C.-P., Silberstein, L., Webster, S. G., Miller, S. C., and Webster, C. (1985) Science 230, 758–766
23. Stone, J., de Lange, T., Ramsay, G., Jakobovits, E., Bishop, J. M., Varmus, H., and Lee, W. (1987) Mol. Cell. Biol. 7, 1697–1709
24. Mcmaster, G. K., and Carmichael, G. C. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 4835–4838
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