MOLECULAR AND CELLULAR BIOLOGY, May 1997, p. 2698–2707 Vol. 17, No. 5
0270-7306/97/$04.00+0

Coding Elements in Exons 2 and 3 Target c-*myc* mRNA Downregulation during Myogenic Differentiation

NEWMAN M. YEILDING AND WILLIAM M. F. LEE*

Department of Medicine and Cancer Center, University of Pennsylvania, Philadelphia, Pennsylvania

Received 3 December 1996/Returned for modification 13 January 1997/Accepted 10 February 1997

Downregulation in expression of the c-*myc* proto-oncogene is an early molecular event in differentiation of murine C2C12 myoblasts into multinucleated myotubes. During differentiation, levels of c-*myc* mRNA decrease 3- to 10-fold despite a lack of change in its transcription rate. To identify *cis*-acting elements that target c-*myc* mRNA for downregulation during myogenesis, we stably transfected C2C12 cells with mutant *myc* genes or chimeric genes in which various *myc* sequences were fused to the human β-globin gene or to the bacterial chloramphenicol acetyltransferase (CAT) gene. Deletion of coding sequences from *myc* exon 2 or exon 3 abolished downregulation of *myc* mRNA during myogenic differentiation, while deletion of introns or sequences in the 5′ or 3′ untranslated regions (UTRs) did not, demonstrating that coding elements in both exons 2 and 3 are necessary for *myc* mRNA downregulation. Fusion of coding sequences from either *myc* exon 2 or 3 to β-globin mRNA conferred downregulation onto the chimeric mRNA, while fusion of *myc* 3′ UTR sequences or coding sequences from CAT or ribosomal protein L32 did not, demonstrating that coding elements in *myc* exons 2 and 3 specifically confer downregulation. These results present the apparent paradox that coding elements in either *myc* exon 2 or *myc* exon 3 are sufficient to confer downregulation onto β-globin mRNA, but neither element alone was sufficient for *myc* mRNA downregulation, suggesting that some feature of β-globin mRNA may potentiate the regulatory properties of *myc* exons 2 and 3. A similar regulatory function is not shared by all mRNAs because fusion of either *myc* exon 2 or *myc* exon 3 to CAT mRNA did not confer downregulation onto the chimeric mRNA, but fusion of the two elements together did. We conclude from these results that two *myc* regulatory elements, one exon 2 and one in exon 3, are required for *myc* mRNA downregulation. Finally, using a highly sensitive and specific PCR-based assay for comparing mRNA levels, we demonstrated that the downregulation mediated by *myc* exons 2 and 3 results in a decrease in cytoplasmic mRNA levels, but not nuclear mRNA levels, indicating that regulation is a postnuclear event.

The c-*myc* proto-oncogene encodes a nuclear phosphoprotein thought to function as a transcription factor that regulates genes important for cell proliferation and differentiation (for a review, see reference 25). c-*myc* expression increases in cells stimulated to proliferate (29), and cells expressing c-*myc* constitutively (1) or microinjected with c-*myc* protein (28) have a decreased requirement for growth factor. Conversely, repression of c-*myc* expression inhibits cell entry into S phase (24). A number of genes thought to encode regulators of cell cycling, including α-prothymosin (17), ornithine decarboxylase (2, 3), cyclin D1 (39), and cdc25A (19), are putative targets of c-Myc transcriptional control. c-*myc* expression decreases in many cells induced to growth arrest and differentiate (15, 30), suggesting a role for c-*myc* downregulation in cell differentiation. This finding is supported by the observations that constitutive c-*myc* expression prevents differentiation (11, 18, 22), while repression of c-*myc* expression induces differentiation (41, 49). These studies suggest that regulated expression of c-*myc* plays a pivotal role in determining cell growth and differentiation behavior.

Both transcriptional (4, 9, 23, 36, 38) and posttranscriptional (6, 13–15, 30, 45) mechanisms regulate c-*myc* expression, depending on the cell type examined and the culture conditions to which the cells are exposed. c-*myc* transcription can be modulated by the activity of upstream promoters and enhancers (21, 33, 36). Alternatively, in many cell lines, including murine fibroblasts (38) and HL60 (23), MEL (36), small-cell lung cancer (31), and B-lymphoid (9) cells, nuclear run-on assays indicate that c-*myc* expression can be regulated by attenuation of transcription near the border of exon 1 and intron 1, resulting in blockade of transcript elongation or premature termination. In other cell lines, such as C2C12 myoblasts (45), Daudi Burkitt's lymphoma cells (13, 30), and F9 teratocarcinoma cells (14, 15), c-*myc* expression is regulated through posttranscriptional mechanisms, presumably acting at the level of mRNA turnover. Critically important for rapid regulation of c-*myc* mRNA is its short half-life (15 to 30 min) (12, 27), which allows cells to rapidly alter c-*myc* expression downward during cell differentiation (6, 13–15, 30, 45) or upward during mitogen stimulation (46).

Regulated RNA stability plays an important role in controlling levels of other mRNAs besides c-*myc* and can occur through a variety of mechanisms. A stem-loop structure in the 3′ untranslated region (UTR) of histone mRNA is necessary for its translation-dependent turnover and tightly couples histone mRNA levels to the cell's position in the cell cycle and DNA synthesis (20). Stem-loop structures termed iron-responsive elements in the 3′ UTR of transferrin receptor mRNA regulate its turnover. Two iron-regulatory proteins that bind to the iron-regulatory elements in an iron-poor environment, stabilize the transferrin receptor mRNA, and increase its steady-state level have been characterized (8, 37, 42); in an iron-rich environment, the unprotected mRNA is rapidly degraded. Autoregulation of β-tubulin mRNA levels depends on translation of its first four codons. Excess free β-tubulin subunits target the β-tubulin mRNA for accelerated turnover through recognition of the encoded tetrapeptide rather than through recog-

* Corresponding author. Mailing address: 663 CRB, 415 Curie Blvd., University of Pennsylvania, Philadelphia, PA 19104. Phone: (215) 898-0258 or (215) 898-0259. Fax: (215) 573-2028. E-mail: leemingf@mail.med.upenn.edu.

nition of the RNA sequence or structure (48). Thus, posttranscriptional regulation of cellular mRNAs can occur through a variety of mechanisms.

We have sought to identify mechanisms by which c-*myc* expression is posttranscriptionally downregulated during C2C12 myogenic differentiation. We previously demonstrated that sequences in the *myc* mRNA 3′ UTR determine its turnover rate and steady-state levels in C2C12 cells under normal growth conditions. However, these sequences are dispensable for downregulation of c-*myc* mRNA levels during differentiation (45, 47). In the present study, we demonstrate that coding sequences in both *myc* exon 2 and *myc* exon 3 are important for its downregulation during myoblast differentiation. We also demonstrate that downregulation occurs in the cytoplasm and not in the nucleus, supporting the idea that regulation occurs by accelerated mRNA turnover.

MATERIALS AND METHODS

Plasmid constructions. Salient features of the plasmids used in our study are diagrammed in Fig. 1. CM19 was previously described (47) and is a pUC-based plasmid that contains all three exons of human c-*myc* from the *Xho*I site (between the P1 and P2 promoters) to the *Eco*RI site (3′ to exon 3) under the transcriptional control of the Moloney murine leukemia virus (MLV) long terminal repeat (LTR). *myc*(X/N) was previously described (47) and contains *myc* sequences from the *Xho*I site to the *Nsi*I site 75 nucleotides (nt) 3′ to the translation termination codon fused to the simian virus 40 T-antigen polyadenylation signal (SVpA). The construction of plasmids *myc*(Δ41-178) and *myc*(Δ265-433), in which *myc* codons 41 to 178 and 265 to 433 were deleted, was previously described (44). *myc* (T/N) was created by ligating the SVpA to the *Nsi*I site of MLV-m23 (45) and contains *myc* cDNA sequences from the *Tha*I site 5 nt upstream of the exon 2 translation initiation codon to the *Nsi*I site. Codons 41 to 178 were deleted from *myc*(T/N) by removing the *Pst*I-*Pst*I fragment to create *myc*(T/N)(Δ41-178). To create *myc*(T/N)(Δ265-433), *myc* codons 265 to 433 were deleted from *myc*(T/N) by substituting the *Cla*I-*Nsi*I fragment of *myc*(Δ265-433).

Construction of the β-globin–*myc* plasmids βGm434SVpA, βGm434*myc*pA, βGm263SVpA, and βGm(40-263)SVpA and their nomenclature were previously described (47). These genes contain an MLV–β-globin backbone encoding the first 140 (of 146) globin amino acids fused to variable *myc* sequences. In βGm434SVpA, MLV–β-globin was fused in frame to *myc* codon 434 and includes the last six *myc* codons and first 75 nt of the *myc* 3′ UTR and uses the SVpA. The other genes were created from βGm434SVpA with the following modifications: in βGm434*myc*pA, the entire *myc* 3′ UTR and flanking sequences to the *Eco*RI site were substituted for the SVpA; in βGm263SVpA, almost all *myc* exon 3 coding sequences (from codon 263 to the *Nsi*I site in *myc* exon 3) were fused in frame to globin; and in βGm(40-263)SVpA, *myc* cDNA sequences from codons 40 to 263 (most exon 2 coding sequences and the first 10 codons in exon 3) were fused in frame to globin. βGm263SVpARI$^-$ was created by introducing a C-to-T mutation by site-directed mutagenesis into β-globin codon 122, which destroyed an *Eco*RI site but preserved the encoded amino acid (phenylalanine). βGm40G was created by substituting the *Xho*I-*Eco*RI fragment of In40 (44) for the same fragment in βGm434SVpA. βGm40G(Δ41-178) was created from βGm40G by removing the *Pst*I-*Pst*I fragment of *myc* exon 2. βGm40G(Δ265-433) was created by substituting the *Cla*I-*Eco*RI fragment of *myc*(Δ265-433) for the same fragment in βGm40G.

In βG-rpL32 and βG-CAT (47), the MLV–β-globin backbone was fused to the first 133 (of a total of 135) codons of ribosomal protein L32 (rpL32) (16) or to the last 181 (of a total of 219) codons of the bacterial gene encoding chloramphenicol acetyltransferase (CAT), respectively; both plasmids use the SVpA.

CAT-*myc* fusion genes were constructed by fusing the *Nco*I-*Eco*RI fragment of β-globin–*myc* plasmids into the *Nco*I-*Bam*HI site of MLV-CAT-SVpA (45) and contain sequences encoding the first 173 CAT amino acids fused in frame to variable *myc* sequences via a short linker with a 5′ *Nco*I site and a 3′ *Xho*I site. (See reference 47 for introduction of an *Nco*I site at β-globin codons 140 and 141.) They were constructed identically to β-globin–*myc* plasmids except that the intronless CAT gene was substituted for the β-globin gene. CAT-*myc* fusion genes were named similarly to β-globin–*myc* genes: in CAT-*myc*(40-439), CAT sequences were fused in frame to *myc* cDNA sequences from codon 40 to the *Nsi*I site followed by the SVpA; CAT-*myc*(40-263) linked *myc* codons 40 to 263 in frame to CAT followed by the SVpA; and CAT-*myc*263 linked in frame *myc* sequences from codon 263 to the *Nsi*I site to CAT followed by the SVpA.

Cell culture and DNA transfection. All experiments were performed with murine C2C12 myoblasts (7) obtained from the American Type Culture Collection (Rockville, Md.) and maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum, 5% CO_2, penicillin, and streptomycin. Plasmids containing test genes were stably cotransfected into C2C12 cells with a plasmid containing a Neo^r gene, using the calcium phosphate method. After selection in 400 μg of G418 (Gibco) per ml, pools of 25 to 50 surviving colonies were expanded for study. Differentiation assays were performed as previously described (45). Briefly, stably transfected C2C12 cells were seeded at subconfluent density into multiple culture plates containing DMEM supplemented with 10% fetal calf serum and antibiotics. Cells were cultured to confluence and then induced to differentiate into multinucleated myotubes by changing the medium to DMEM containing 2% horse serum.

Analysis of mRNA levels during differentiation in stably transfected cells. Total cytoplasmic mRNA was isolated on serial culture days as previously described (47), and mRNA levels were determined by Northern analysis using the glyoxal method (35). RNA was electroblotted to Hybond N (Amersham) and UV cross-linked. Hybridizations were carried out by modifications of the method of Church and Gilbert (10), using probes labeled by random priming. Human *myc* mRNAs were probed with a human c-*myc* exon 1 probe (*Xho*I-to-*Pvu*II fragment) or a human c-*myc* exon 2+3 cDNA probe from pSP65*myc*IIA (44). β-Globin chimeric mRNAs were probed with a full-length human β-globin cDNA fragment from pSPβκc (gift from Stephen Liebhaber). CAT chimeric mRNAs were probed with the *Hin*dIII-*Bam*HI fragment of MLV-CAT-SVpA (45). C2C12 c-*myc* mRNA was probed with a murine c-*myc* exon 1 probe (*Bam*HI-to-*Sac*I fragment) or a human c-*myc* exon 2+3 cDNA probe from pSP65*myc*IIA (44). rpL32 mRNA was probed with a full-length cDNA probe (16). Northern blots were analyzed on a Molecular Dynamics (Sunnyvale, Calif.) PhosphorImager using ImageQuant software, and the relative levels of *myc*, globin-*myc*, and CAT-*myc* mRNAs during differentiation were determined by normalizing for RNA loading by reference to the level of rpL32 mRNA unless otherwise stated. A decrease in endogenous c-*myc* mRNA levels by at least threefold after cells reached confluence and were induced to differentiate was considered to signify that *myc* mRNA downregulation had occurred, and mRNA from the transgene was considered downregulated if its normalized level decreased at least threefold during differentiation. The Northern blots shown are representative of the results obtained on each stably transfected cell line. The bar graphs shown depict the levels of *myc*, globin-*myc*, and CAT-*myc* mRNAs relative to mRNA levels in preconfluent, undifferentiated cells after normalization for RNA loading by reference to the level of rpL32 mRNA unless otherwise stated. The Bio-Rad (Hercules, Calif.) photodocumentation system and Molecular Analyst software were used to quantitate relative RNA loading, using ethidium bromide-stained gels.

RT-PCR+1 assay of comparative mRNA abundance. The reverse transcription (RT)-PCR+1 assay for comparing the abundances of two β-globin-containing mRNAs was performed as previously described (47), with minor modifications. Total cytoplasmic RNA was extracted from transfected C2C12 cells as previously described. To isolate nuclear RNA, nuclei were isolated after C2C12 cell lysis in nuclear lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 5 mM $MgCl_2$, 0.5% Nonidet P-40). After nuclei were washed in nuclear lysis buffer, nuclear RNA was isolated by using Trizol Reagent (Gibco BRL). Sequence-specific reverse transcription of cytoplasmic and nuclear RNAs was performed by priming with oligonucleotide βG3′Rev (see below), using M-MLV reverse transcriptase (Gibco BRL). PCR amplification of cDNA was performed with *Taq* polymerase (Promega) in 50 mM Tris-HCl (pH 9.0)–20 mM $(NH_4)_2SO_4$–1.5 mM $MgCl_2$–200 mM deoxynucleoside triphosphates–1 mM oligonucleotide primers on a PTC-100 thermal cycler (MJ Research, Cambridge, Mass.). A 214-bp sequence of β-globin cDNA was amplified by using oligonucleotide primers βGF2 and βG3′Rev (see below) under the following conditions: 30 cycles of 92°C for 30 s, 62°C for 15 s, and 72°C for 15 s. A +1 cycle was conducted with a ^{32}P-end-labeled nested primer, βGF1, and the products were restricted with *Eco*RI and resolved by electrophoresis. A radiolabeled RT-PCR-generated 329-bp DNA fragment containing a unique *Eco*RI site was added to each *Eco*RI digestion reaction to indicate complete digestion of the RT-PCR+1 products. The relative abundances of the RT-PCR+1 products, and hence of the mRNAs, were quantitated on a Molecular Dynamics PhosphorImager using ImageQuant software. The primers used in this study were βGF2 (5′AAGTGCTCGGTGCC TTTAGTGA3′), βG3′Rev (5′ACACCAGCCACCACTTTCTGA3′), and βGF1 (5′CAAGGGCACCTTTGCCACACT3′).

RESULTS

Protein coding sequences in exons 2 and 3 are necessary for downregulation of c-*myc* mRNA in differentiating C2C12 cells. When murine C2C12 myoblasts are induced to differentiate into multinucleated myotubes by culturing to confluence and changing to mitogen-poor culture media, the level of c-*myc* mRNA is downregulated over 12 to 24 h (Fig. 2) through posttranscriptional mechanisms (45). While the mRNAs of housekeeping genes such as those encoding rpL32 and glyceraldehyde-3-phosphate dehydrogenase can be variably downregulated (0 to 50%, normalized to 18S and 28S RNA levels on ethidium bromide-stained gels), c-*myc* mRNA is downregulated 3- to 10-fold compared to these housekeeping mRNAs. The magnitude of c-*myc* mRNA downregulation generally cor-

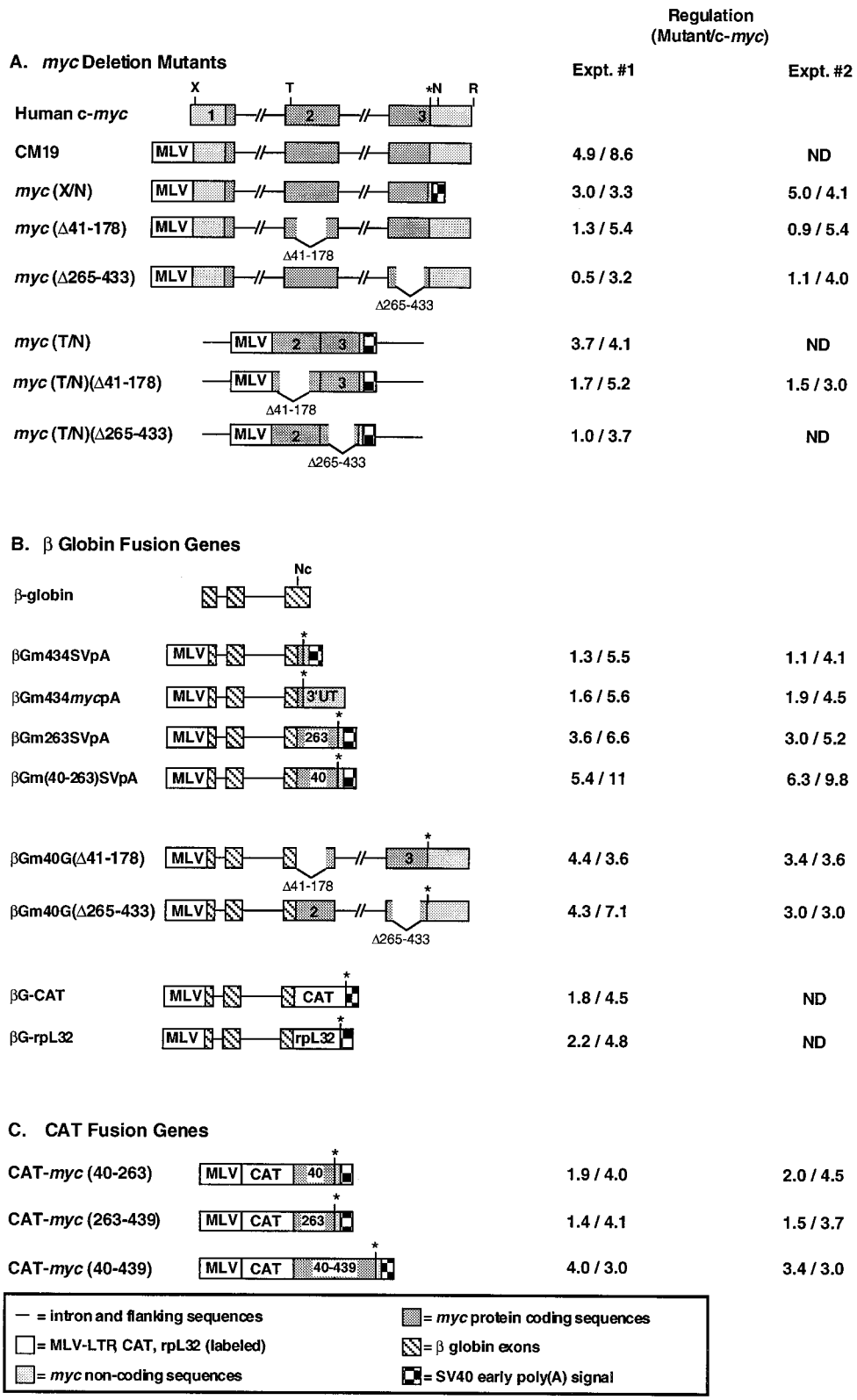

FIG. 1. Schematic diagram of recombinant genes used in these studies. Construction of these genes is outlined in Materials and Methods. The structural features of these genes and the shading patterns of exonic sequences are indicated at the bottom. An *Nco*I restriction site was introduced into exon 3 of MLV–β-globin, which allowed construction of chimeric β-globin genes. Endonuclease restriction sites used in construction are indicated as follows: *Nsi*I, N; *Nco*I, Nc; *Eco*RI, R; *Tha*I, T; and *Xho*I, X. The *Nco*I site in MLV–β-globin exon 3 was introduced by site-directed mutagenesis (47). 40 and 263 designate *myc* coding sequences from codons 40 to 263 and from codon 263 to the *Nsi*I site 75 nt downstream of the translation termination codon, respectively. The translation termination codon in each construct is depicted by an asterisk. These constructs were stably transfected into C2C12 cells, and downregulation of their mRNAs during myoblast differentiation was determined as outlined in Materials and Methods. The maximum fold downregulation of their mRNAs is shown to the right of each construct and compared to the fold downregulation of endogenous murine c-*myc* mRNA. 3′UT, 3′ UTR; SV40, simian virus 40.

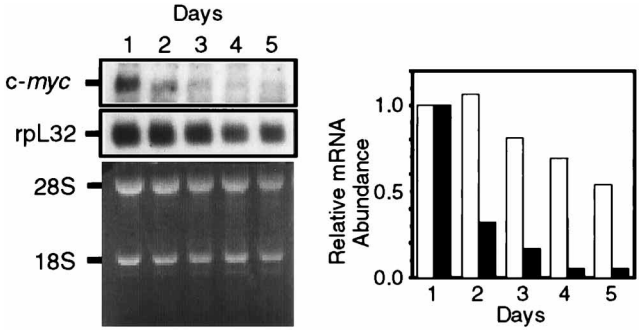

FIG. 2. c-*myc* mRNA is markedly downregulated during C2C12 myogenic differentiation. C2C12 cells were seeded at low density into multiple culture plates, cultured to confluence, and induced to differentiate in differentiation medium (DMEM containing 2% horse serum). Northern analysis was performed on cytoplasmic RNA isolated on serial days beginning the day after cells were plated. Cells were subconfluent on day 1 and confluent on day 2. The medium was changed to differentiation medium on day 2; therefore, day 3, 4, and 5 cells had been exposed to differentiation medium for 1, 2, and 3 days, respectively. The left panel displays an autoradiograph of a Northern blot probed for c-*myc* mRNA by using a murine c-*myc* exon 1 probe (*Bam*HI-to-*Sac*I fragment) and for rpL32 mRNA by using a full-length cDNA probe (16). The ethidium bromide-stained agarose gel is shown below the autoradiographs. The right panel displays the relative abundance of c-*myc* and rpL32 mRNAs compared to their levels in preconfluent cells. Levels shown were normalized to ethidium bromide staining to correct for RNA loading.

relates with the percentage of cells that undergo morphological differentiation with greater than threefold downregulation seen in cultures in which the majority of cells have morphologically differentiated (unpublished observations). As shown in Fig. 2, levels of c-*myc* mRNA were markedly downregulated as cells reached confluence (day 2 of culture) and continued to decrease after cells were changed to mitogen-poor medium on day 2. This continued decrease in levels of c-*myc* mRNA likely results from asynchronous differentiation. We previously demonstrated that a transfected full-length human c-*myc* gene or a human c-*myc* gene under the transcriptional control of the MLV LTR encodes an mRNA that is regulated like endogenous murine c-*myc* mRNA when these cells are induced to differentiate (45). We sought to identify the *cis*-acting elements targeting c-*myc* mRNA for downregulation.

As a first step in the identification of regulatory elements, we analyzed the regulation of human *myc* mRNAs containing deletion mutations. C2C12 cells were stably transfected with (i) CM19, an almost full-length human c-*myc* gene; (ii) *myc*(X/N), a mutant human *myc* gene from which most 3′ UTR sequences were deleted; (iii) *myc*(Δ265-433), a mutant from which most exon 3 coding sequences were deleted; or (iv) *myc*(Δ41-178), a mutant from which most of exon 2 was deleted. Twenty-five to 50 clones were pooled for analysis to ensure that clonal variation did not skew results. Cytoplasmic RNA was isolated on serial days from subconfluent, undifferentiated cells, confluent cells, and cells induced to differentiate. Northern analysis demonstrated that mRNAs from CM19 and *myc*(X/N) were maximally downregulated 4.9- and 3.0-fold, respectively, during C2C12 differentiation, similar to the 8.6- and 3.3-fold downregulation of endogenous c-*myc* mRNA in the respective cells (all normalized to rpL32 mRNA) (Fig. 1 and 3A and B). The modest increase in levels of c-*myc* and CM19 mRNAs seen in later cultures (Fig. 3A, culture days 4 and 5) likely represents outgrowth of undifferentiated cells. In contrast, levels of *myc*(Δ41-178) and *myc*(Δ265-433) mRNAs were essentially unchanged or increased slightly during differentiation (Fig. 1 and 3C and D). Endogenous c-*myc* mRNA was downregulated 5.4- and 3.2-fold in *myc*(Δ41-178) and *myc*(Δ265-433) stable transfectants, respectively, demonstrating that the cellular regulatory machinery was mobilized. Repeat differentiation assays yielded similar results (Fig. 1), leading to the conclusion that both exon 2 and exon 3 coding sequences are necessary for *myc*

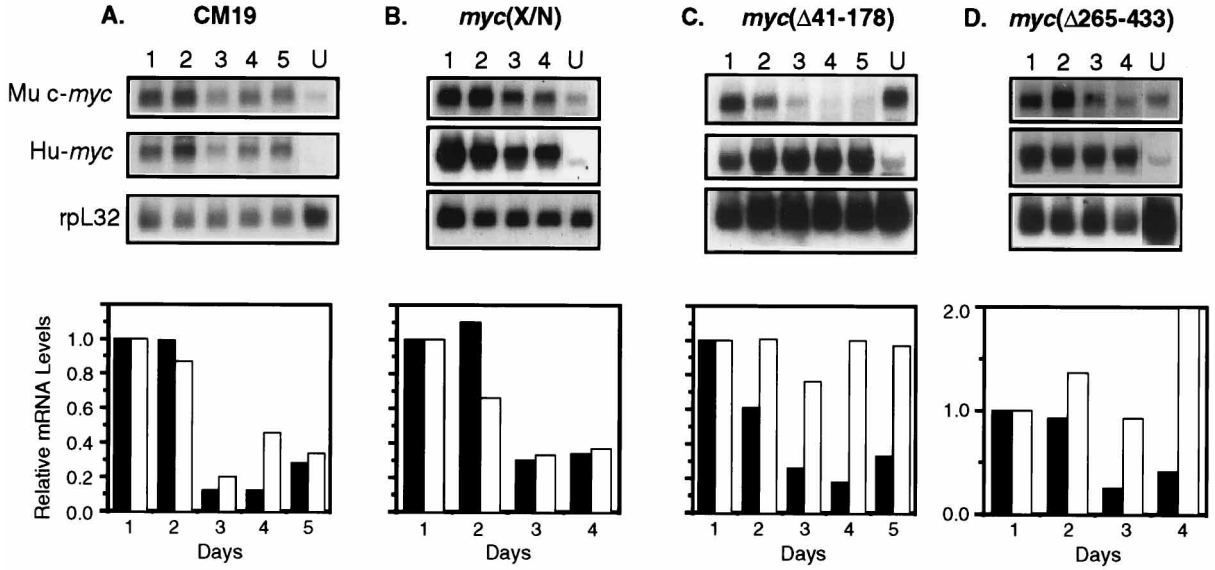

FIG. 3. Deletion of coding sequences from *myc* exon 2 or 3, but not 3′ UTR sequences, abolishes *myc* mRNA downregulation during myoblast differentiation. Differentiation assays were conducted on C2C12 cells stably transfected with CM19, *myc*(X/N), *myc*(Δ41-178), or *myc*(Δ265-433). (See the legend in Fig. 2 for details of the assay.) Autoradiographs of Northern blots display mRNA levels in cells transfected with CM19 (A), *myc*(X/N) (B), *myc*(Δ41-178) (C), or *myc* (Δ265-433) (D). mRNA from the transfected gene was detected by using a human c-*myc* exon 1 probe (*Xho*I-to-*Pvu*II fragment) (Hu-myc); the endogenous murine (Mu) c-*myc* mRNA was detected by using a murine c-*myc* exon 1 probe; and rpL32 mRNA was detected by using a full-length cDNA probe (16). RNA from untransfected C2C12 cells (lanes U) was used to demonstrate specificity of the human c-*myc* exon 1 probe. The bar graphs depict the levels of endogenous murine c-*myc* mRNA (dark bars) and transgene mRNA (light bars) relative to their mRNA levels in preconfluent, undifferentiated cells after normalization for RNA loading by reference to the level of rpL32 mRNA.

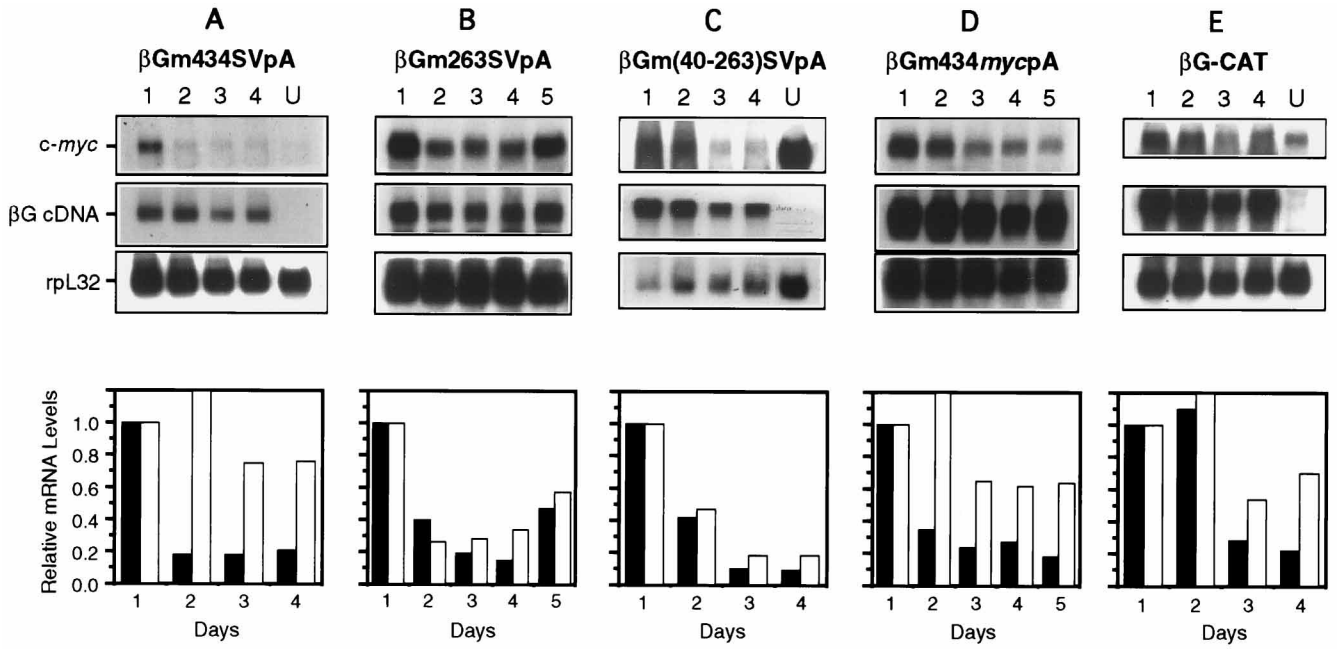

FIG. 4. Coding sequences from *myc* exon 2 or 3, but not *myc* 3′ UTR or CAT sequences, confer downregulation on β-globin during myoblast differentiation. Differentiation assays were conducted on C2C12 cells stably transfected with βGm434SVpA, βGm(40-263)SVpA, βGm263SVpA, βGm434*myc*pA, or βG-CAT. (See the legend in Fig. 2 for details of the assay.) Autoradiographs of Northern blots display mRNA levels in cells transfected with βGm434SVpA (A), βGm(40-263)SVpA (B), βGm263SVpA (C), βGm434*myc*pA (D), and βG-CAT (E). mRNA from the transfected gene was detected by using a full-length human β-globin (βG) cDNA probe, pSPβκc; endogenous murine c-*myc* and rpL32 mRNAs were detected by using *myc* exon 2 and 3 and full-length rpL32 probes, respectively. RNA from untransfected C2C12 cells (lanes U) was used to demonstrate specificity of the human β-globin cDNA probe. The bar graphs depict the levels of endogenous murine c-*myc* mRNA (dark bars) and transgene mRNA (light bars) relative to their mRNA levels in preconfluent, undifferentiated cells after normalization for RNA loading by reference to the level of rpL32 mRNA.

mRNA downregulation during differentiation, but 3′ UTR sequences are dispensable.

Fusion of protein coding sequences from *myc* exon 2 or 3 to globin confers downregulation on the chimeric mRNA during C2C12 differentiation. To identify *myc* sequences that target the mRNA for downregulation, we identified those that could confer regulation on human β-globin mRNA. C2C12 cells were stably transfected with βGm434SVpA, βGm(40-263)SVpA, or βGm263SVpA to determine if coding sequences from *myc* exon 2 or 3 alone could confer regulation onto β-globin mRNA. Levels of βGm434SVpA mRNA decreased only 1.3-fold during C2C12 differentiation, compared to a 5.5-fold downregulation of endogenous c-*myc* mRNA (Fig. 1 and 4A), which showed that β-globin mRNA fused in frame to *myc* sequences from codon 434 to the *Nsi*I site is insufficient for downregulation. Surprisingly, both βGm(40-263)SVpA and βGm263SVpA mRNAs were downregulated during differentiation (5.4- and 3.6-fold, respectively, compared to 11.0- and 6.6-fold downregulation of endogenous c-*myc* mRNA) (Fig. 1 and 4B and C). This result demonstrated that coding sequences in *myc* exon 2 and 3 could independently downregulate β-globin mRNA.

To exclude the possibility that downregulation of βGm(40-263)SVpA and βGm263SVpA mRNAs was a nonspecific effect of fusing additional sequences to globin mRNA, C2C12 cells were stably transfected with βGm434*myc*pA, βG-CAT, or βG-rpL32. Northern analyses of RNA isolated from these transfectants during differentiation demonstrated that *myc* 3′ UTR sequences or most of the coding region from CAT or rpL32 conferred no more than 2.2-fold downregulation of globin mRNA (Fig. 1 and 4D and E). This result showed that downregulation of βGm(40-263)SVpA and βGm263SVpA mRNAs was specifically conferred by *myc* exon 2 or 3 coding sequences. This finding, together with the results of the *myc* deletion mRNA studies, present the curious situation where sequences from both *myc* exons 2 and 3 are needed to downregulate *myc* mRNA, while sequences from either exon alone can confer downregulation when fused to β-globin mRNA.

***myc* intron and noncoding mRNA sequences do not inhibit mRNA downregulation.** The apparent paradox of *myc* and globin-*myc* mRNA downregulation might be explained by the presence of regulation-inhibitory sequences in *myc* mRNA in addition to the regulatory elements in exons 2 and 3. Such inhibitory sequences could neutralize the regulatory effects of exon 2 or 3 sequences when either element is present alone. To determine whether inhibitory sequences in other regions of *myc* mRNA prevent coding sequences from either *myc* exon 2 or *myc* exon 3 alone from conferring downregulation, C2C12 cells were stably transfected with *myc*(T/N), *myc*(T/N)(Δ41-178), and *myc*(T/N)(Δ265-433). These plasmids contain truncated *myc* cDNAs from which *myc* introns and almost all 5′ and 3′ noncoding sequences have been deleted. During C2C12 differentiation, *myc*(T/N) mRNA was downregulated 3.7-fold, similar to the 4.1-fold downregulation of endogenous c-*myc* mRNA (Fig. 1 and 5A). This result shows that only the *myc* coding domain (and not 5′ and 3′ UTR and intron sequences) is necessary for normal *myc* mRNA downregulation. In contrast, neither *myc*(T/N)(Δ41-178) nor *myc*(T/N)(Δ265-433) mRNA was downregulated (Fig. 1 and 5B and C), which shows that coding sequences from both *myc* exons 2 and 3 are needed for downregulation, even when other portions of *myc* mRNA are absent.

To test whether *myc* intron or UTR sequences inhibit downregulation of β-globin mRNA fused to *myc* exon 2 or exon 3

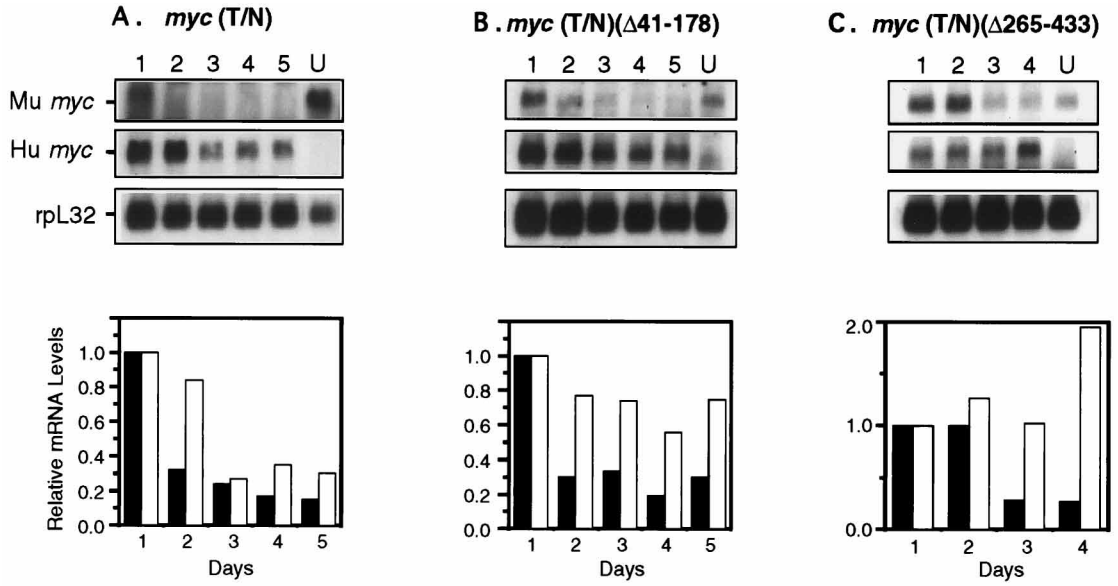

FIG. 5. Deletion of introns and UTRs does not affect regulation of *myc* mRNAs. Differentiation assays were conducted on C2C12 cells stably transfected with *myc*(T/N), *myc*(T/N)(Δ41-178), or *myc*(T/N)(Δ265-433). (See the legend in Fig. 2 for details of the assay.) Autoradiographs of Northern blots display mRNA levels in cells transfected with *myc*(T/N) (A), *myc*(T/N)(Δ41-178) (B), or *myc*(T/N)(Δ265-433) (C). mRNA from the transfected gene was detected by using a human (Hu) c-*myc* exon 2 and 3 probe; the endogenous murine (Mu) c-*myc* mRNA was detected by using a murine c-*myc* exon 1 probe; and rpL32 mRNA was detected by using a full-length cDNA probe (16). RNA from untransfected C2C12 cells (lanes U) was used to demonstrate specificity of the human c-*myc* exon 2 and 3 probe. The bar graphs depict the levels of endogenous murine c-*myc* mRNA (dark bars) and transgene mRNA (light bars) relative to their mRNA levels in preconfluent, undifferentiated cells after normalization for RNA loading by reference to the level of rpL32 mRNA.

sequences, C2C12 cells were stably transfected with βGm40G(Δ41-178) and βGm40G(Δ265-433). These plasmids contain globin fused in frame to coding sequences from *myc* exon 2 or 3 with *myc* intron 2 and 3′ UTR sequences present. The sizes of their mRNAs on Northern blots indicated that these mRNAs were correctly spliced. During C2C12 differentiation, βGm40G(Δ41-178) and βGm40G(Δ265-433) mRNAs decreased 4.4- and 4.3-fold, respectively (Fig. 1 and 6), which was comparable to the downregulation of endogenous c-*myc* mRNA. Combined with the studies of mRNAs from *myc*(T/N) derivatives, these results show that *myc* intron and UTR sequences do not inhibit mRNA downregulation. Therefore, the presence of these elements does not explain why sequences from both exons 2 and 3 are necessary for *myc* mRNA downregulation, and their absence does not explain why globin mRNA is regulated when fused to either exon 2 or 3 sequences.

Multiple regulatory elements are necessary for mRNA downregulation during differentiation. An alternative explanation for the conundrum seen with regulation of *myc* and β-globin–*myc* mRNA is that some property of β-globin mRNA potentiates the regulatory effects of the *myc* coding exons. If this is the case, fusion of *myc* exon 2 or 3 sequences to another heterologous mRNA might produce results different from those for globin-*myc* fusion mRNAs. A globin-CAT fusion mRNA was not regulated during C2C12 cell differentiation (Fig. 1 and 4E), suggesting that CAT mRNA does not contain a regulatory element. Therefore, we made CAT-*myc*(40-263), CAT-*myc*(263-439), and CAT-*myc*(40-439), which contain CAT sequences fused in frame to coding sequences from *myc* exon 2, exon 3, or exons 2 and 3, sequentially, and transfected them into C2C12 cells. During C2C12 differentiation, neither CAT-*myc*(40-263) nor CAT-*myc*(263-439) mRNA was downregulated significantly (Fig. 1 and 7A and B). However, CAT-*myc*(40-439) mRNA was downregulated 4.0-fold (Fig. 1 and 7C), demonstrating that *myc* exons 2 and 3 together, but neither alone, confer regulation on CAT mRNA. Therefore, some property of or sequence in β-globin mRNA that is not shared by CAT mRNA complements the functionality of *myc* mRNA regulatory elements in exons 2 and 3 and allows each to impart mRNA downregulation when fused to β-globin mRNA individually.

mRNA downregulation mediated by *myc* exon 2 or 3 occurs as a postnuclear event. Previous studies have demonstrated that downregulation of c-*myc* mRNA during C2C12 differentiation occurs posttranscriptionally (45), but whether it occurs in the nucleus or in the cytoplasm is unknown. To determine whether nuclear as well as cytoplasmic mRNA levels are altered during downregulation, we used an assay for comparing the relative abundances of two globin-*myc* fusion mRNAs. Previously, we demonstrated that the levels of two globin-*myc* fusion mRNAs can be compared by a sensitive and specific RT-PCR+1 assay (47). After RT-PCR amplification of a 214-bp sequence of the globin-*myc* fusion mRNAs, an additional cycle of amplification is conducted with a radiolabeled nested primer. The product from one of the two mRNAs contains a unique *Eco*RI site, allowing the two products to be distinguished after digestion.

To determine whether *myc* exon 3-mediated mRNA downregulation is a nuclear or cytoplasmic event, C2C12 cells were stably cotransfected with βGm263SVpARI$^-$ and βGm434SVpA; the former contains β-globin fused to *myc* exon 3 coding sequences and encodes an mRNA downregulated during differentiation, while the latter encodes an unregulated mRNA. The comparative abundances of spliced βGm434SVpA and βGm263SVpARI$^-$ mRNAs in a nuclear RNA preparation were determined by RT-PCR+1 analysis. A single 168-nt band was produced by RT-PCR+1 of nuclear RNA from both undifferentiated and differentiated cells (Fig. 8A, lanes 9 and 10), while control reactions were negative (Fig.

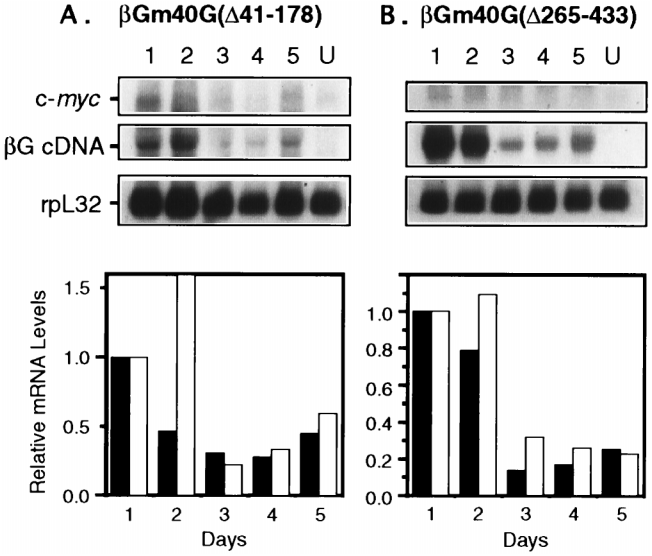

FIG. 6. *myc* intron 2 and 3′ UTR sequences do not inhibit mRNA downregulation during myoblast differentiation. Differentiation assays were conducted on C2C12 cells stably transfected with βGm40G(Δ41-178) or βGm40G(Δ265-433). (See the legend in Fig. 2 for details of the assay.) Autoradiographs of Northern blots display mRNA levels in cells transfected with βGm40G(Δ41-178) (A) and βGm40G(Δ265-433) (B). mRNA from the transfected gene was detected by using a full-length human β-globin (βG) cDNA probe, pSPβκc; endogenous murine c-*myc* and rpL32 mRNAs were detected by using *myc* exon 2 and 3 and full-length rpL32 probes, respectively. RNA from untransfected C2C12 cells (lanes U) was used to demonstrate specificity of the human β-globin cDNA probe. The bar graphs depict the levels of endogenous murine c-*myc* mRNA (dark bars) and transgene mRNA (light bars) relative to their mRNA levels in preconfluent, undifferentiated cells after normalization for RNA loading by reference to the level of rpL32 mRNA.

8A, lane 2). Product derived from βGm434SVpA mRNA contains an *Eco*RI site, and the radiolabeled portion is restricted to 119 nt after *Eco*RI digestion, while product from βGm263SVpARI$^-$ mRNA is not cut by *Eco*RI and remains at 168 nt. After *Eco*RI digestion, the ratio of spliced nuclear βGm434SVpA mRNA to βGm263SVpARI$^-$ mRNAs was found to be 1.5:1 in both undifferentiated and differentiated C2C12 cells (Fig. 8A, lanes 5 and 6). In contrast, the ratio of βGm434SVpA mRNA to βGm263SVpARI$^-$ mRNA in the cytoplasm was 3.0:1 in undifferentiated cells (Fig. 8A, lanes 3 and 7), which suggests a twofold-higher cytoplasmic turnover rate for βGm263SVpARI$^-$ than βGm434SVpA mRNA (when the 3:1 cytoplasmic mRNA ratio is compared to the 1.5:1 nuclear ratio). This finding is consistent with our previous observations that fusion of *myc* exon 3 coding sequences destabilizes β-globin mRNA (47). After cells were induced to differentiate, the comparative abundance in the cytoplasm changed to 8.4:1 (Fig. 8A, lanes 4 and 8), indicating an almost threefold downregulation of βGm263SVpARI$^-$ mRNA compared to βGm434SVpA mRNA and a five- to sixfold-higher comparative turnover rate. Therefore, *myc* exon 3-mediated mRNA downregulation affects relative cytoplasmic mRNA levels but does not affect relative nuclear mRNA levels.

To determine whether *myc* exon 2 mediates mRNA downregulation in the nucleus or cytoplasm, βGm(40-263)SVpARI$^-$ and βGm434SVpA were stably cotransfected into C2C12 cells. RT-PCR+1 analysis of nuclear RNA demonstrated that the ratios of spliced nuclear βGm434SVpA mRNA to βGm(40-263)SVpARI$^-$ mRNA were similar in undifferentiated and differentiated cells (1:1 and 1:1.1; Fig. 8B, lanes 5 and 6). In the cytoplasm, the ratios were 1.1:1 in undifferentiated cells but 3.3:1 in differentiated cells (Fig. 8B, lanes 3 and 4). The greater comparative abundance of cytoplasmic βGm434SVpA mRNA compared to βGm(40-263)SVpARI$^-$ mRNA following differentiation indicates a threefold selective downregulation of βGm(40-263)SVpARI$^-$ mRNA, probably due to a threefold-higher cytoplasmic turnover rate. These results also demonstrate that both *myc* exon 2- and exon 3-mediated mRNA downregulation affects cytoplasmic mRNA levels but not nuclear mRNA levels.

DISCUSSION

Posttranscriptional mechanisms have been shown to play an important role in regulating c-*myc* expression (6, 13–15, 30, 45). We have been studying the mechanism by which c-*myc* mRNA is posttranscriptionally downregulated during C2C12 differentiation. While housekeeping gene mRNAs (e.g., rpL32 and glyceraldehyde-3-phosphate dehydrogenase gene mRNAs) can be modestly downregulated early in myoblast differentiation, c-*myc* mRNA is specifically targeted for much greater downregulation. Our studies identified *cis*-acting elements that target *myc* mRNA for downregulation. Using C2C12 cells stably transfected with mutant *myc* genes, we showed that removal of coding sequences from either exon 2 or exon 3, but not removal of introns or UTR sequences, abolished *myc* mRNA downregulation during differentiation. Fusion of either *myc* exon 2 or exon 3 coding sequences, but not *myc* 3′ UTR, CAT, or rpL32 sequences, conferred downregulation on β-globin mRNA, an mRNA that is normally not downregulated. Interestingly, neither *myc* exon 2 nor exon 3 coding sequences were sufficient to confer regulation onto CAT mRNA, but fusion of both exons did confer downregulation. Finally, using an assay that compares the relative abundances of two mRNAs, we demonstrated that the downregulation mediated by *myc* coding sequences occurs in the cytoplasm and not in the nucleus.

Our results suggest that two regulatory elements, one in *myc* exon 2 and the other in exon 3, are necessary for normal *myc* mRNA downregulation during myoblast differentiation. Analyses of regulation of *myc* mRNAs and globin-*myc* fusion mRNAs presented a conundrum in which coding sequences from either *myc* exon alone downregulated globin mRNA but did not downregulate c-*myc* mRNA; both elements were necessary for *myc* mRNA downregulation. Our studies excluded the existence of a network of regulatory elements in c-*myc* mRNA, some having positive and some having negative modulatory effects. Putative negative modulatory elements would have to reside in exon 1, UTR, or intron sequences (i.e., outside regions in exon 2 and 3 that were capable of downregulating globin-*myc* fusion mRNAs). Analyses of mRNAs encoded by the *myc*(T/N) genes lacking exon 1, UTR, and intron sequences and by globin-*myc* fusion genes containing intron 2 and 3′ UTR sequences failed to reveal inhibitory elements. Therefore, this puzzle is best explained by some feature of globin mRNA which enables it to complement or enhance the function of each of the *myc* regulatory elements. The special nature of globin mRNA is supported by the behavior of chimeric CAT mRNAs which, unlike chimeric globin mRNAs, require both *myc* exons 2 and 3 to be fused for regulation. In addition, we previously demonstrated that using globin mRNA as a fusion partner can reveal the mRNA destabilizing effect of *myc* exon 3 sequences which is inapparent in its authentic *myc* context in proliferating C2C12 cells (47). These results invite caution in the interpretation of results obtained with chimeric mRNAs, since unsuspected regulatory functions may be unmasked when mRNAs thought to be inert are fused to heter-

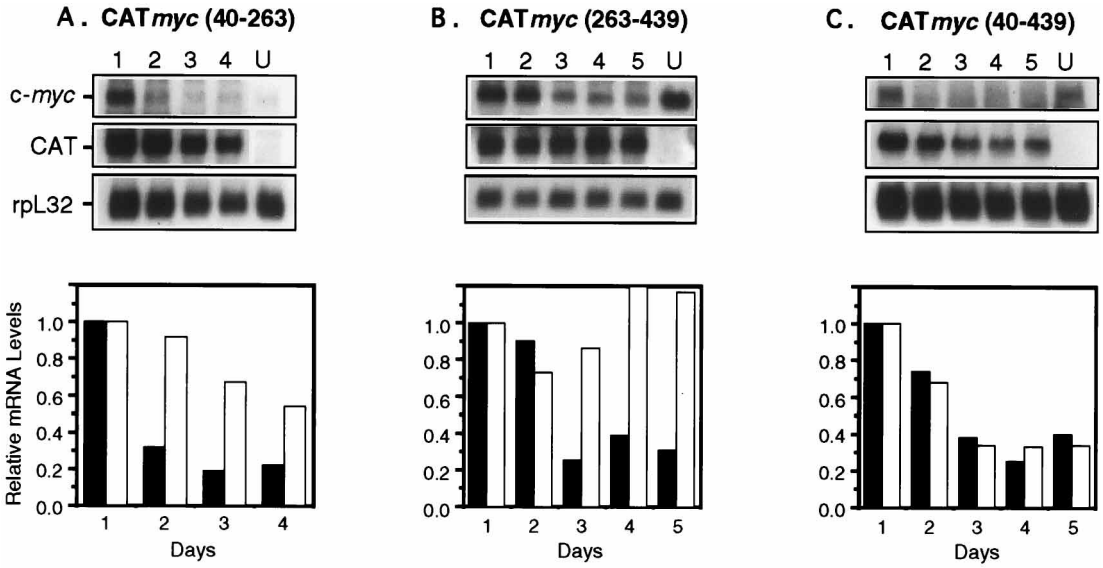

FIG. 7. Coding sequences from both *myc* exons 2 and 3 are necessary to confer regulation onto CAT mRNA during myoblast differentiation. Differentiation assays were conducted on C2C12 cells stably transfected with CAT-*myc*(40-263), CAT-*myc*(263-439), or CAT-*myc*(40-439). (See the legend in Fig. 2 for details of the assay.) Autoradiographs of Northern blots display mRNA levels in cells transfected with CAT-*myc*(40-263) (A), CAT-*myc*(263-439) (B), and CAT-*myc*(40-439) (C). mRNA from the transfected gene was detected by using a full-length CAT cDNA probe; endogenous murine c-*myc* and rpL32 mRNAs were detected by using a murine *myc* exon 1 probe and full-length rpL32 probe, respectively. RNA from untransfected C2C12 cells (lanes U) was used to demonstrate specificity of the CAT cDNA probe. The bar graphs depict the levels of endogenous murine c-*myc* mRNA (dark bars) and transgene mRNA (light bars) relative to their mRNA levels in preconfluent, undifferentiated cells after normalization for RNA loading by reference to the level of rpL32 mRNA.

ologous sequences or placed in a different context. We point out, however, that *myc* mRNA regulatory functions revealed through fusion to globin mRNA have not been false. Rather, globin mRNA fusion seems to accentuate or exaggerate the regulatory function of segments of *myc* mRNA; thus far, we have been able to verify the function of these segments by other means.

It is unclear how exon 2 and 3 coding sequences modulate regulation during differentiation and whether these elements are targeted by independent mechanisms or by a single com-

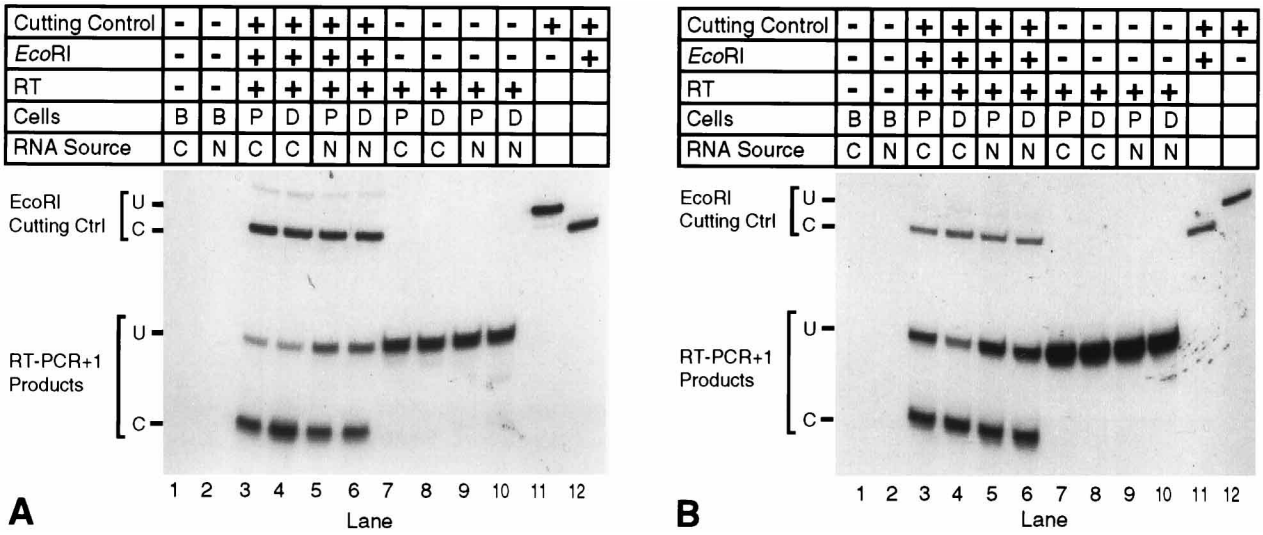

FIG. 8. *myc* exons 2 and 3 mediate downregulation in the cytoplasm. C2C12 cells were stably cotransfected with βGm263SVpARI$^-$ and βGm434SVpA or with βGm(40-263)SVpARI$^-$ and βGm434SVpA. Cytoplasmic (C) and nuclear (N) RNAs were extracted from preconfluent cells (P) and from cells allowed to grow to confluence and induced to differentiate for 2 days in differentiation medium (D). Comparative levels of mRNAs from the β-globin-*myc* genes were determined by RT-PCR+1, and the products were resolved on a 6% denaturing polyacrylamide gel. Autoradiographs display the following RT-PCR+1 products: undigested RT-PCR+1 products (lanes 1, 2, and 7 to 11) and *Eco*RI-digested RT-PCR+1 products (lanes 3 to 6 and 12). *Eco*RI-digested RT-PCR+1 products were spiked with the *Eco*RI cutting control prior to digestion. Digested and undigested *Eco*RI cutting controls are shown in lanes 11 and 12. Results of PCR+1 amplification of RNA from both (B) preconfluent and differentiating cells that has not been reverse transcribed are shown in lanes 1 and 2. *Eco*RI and RT-PCR+1 cut (C) and uncut (U) products are labeled. Results from C2C12 cells cotransfected with βGm263SVpARI$^-$ and βGm434SVpA are shown in panel A, and those from C2C12 cells cotransfected with βGm(40-263)SVpARI$^-$ and βGm434SVpA are shown in panel B.

mon mechanism. We previously demonstrated that the rate of c-*myc* transcription from its own or the MLV LTR promoter is unchanged during C2C12 differentiation (45). Therefore, mRNA downregulation is likely to occur through either accelerated mRNA decay or altered nuclear mRNA processing. We could address the issue of the cellular location of *myc* mRNA downregulation through use of a highly sensitive and specific RT-PCR-based method for comparing the levels of two mRNAs by using a reference mRNA that is not downregulated during C2C12 differentiation. In undifferentiated C2C12 cells, comparative levels of a globin-*myc* fusion mRNA containing the exon 2 regulatory element and a globin-*myc* fusion mRNA lacking either *myc* regulatory element were identical in the nucleus and cytoplasm, suggesting that their cytoplasmic turnover rates are similar. After cells were induced to differentiate, the relative nuclear levels of their spliced mRNAs did not change, but the cytoplasmic level of the *myc* exon 2-containing fusion mRNA markedly decreased relative to the level of the fusion mRNA lacking *myc* regulatory elements. Considered with the unchanged rate of transcription (45), these results demonstrate that downregulation during C2C12 differentiation is a postnuclear event and suggest that turnover of *myc* exon 2-containing mRNAs is accelerated after differentiation. Our assay is unable to distinguish whether this occurs in the cytoplasm or during nuclear-cytoplasmic transport of the mRNA.

The cellular location of *myc* exon 3-mediated mRNA downregulation was similarly identified. Unlike *myc* exon 2, exon 3 destabilizes a globin-*myc* fusion mRNA even in undifferentiated cells. Compared to a globin-*myc* fusion mRNA lacking any *myc* regulatory element, *myc* exon 3-containing globin fusion mRNA was twofold less abundant in the cytoplasm than in the nucleus of undifferentiated C2C12 cells, suggesting that its cytoplasmic turnover rate is faster. After differentiation, the nuclear mRNA ratio is unchanged, but the drop in cytoplasmic mRNA ratio becomes more pronounced, demonstrating that *myc* exon 3-mediated downregulation, like that of exon 2, is a postnuclear event probably resulting from accelerated mRNA decay. While it is unclear whether exons 2 and 3 contain simply redundant regulatory elements or are targeted by independent mechanisms, our results demonstrate that both elements target mRNA for downregulation through postnuclear events.

Our results suggest that sequences from *myc* exon 2 and 3 likely mediate mRNA downregulation by destabilizing the mRNA under differentiation conditions. Coding sequences from *myc* exon 3 were previously implicated as a destabilizing element through in vitro mRNA decay assays (5, 26) and through study of c-*myc* mRNA induction after inhibiting translation with cycloheximide (46). After treatment of cells with cycloheximide, levels of c-*myc* mRNA are rapidly and markedly induced. There is little change in the rate of c-*myc* transcription, and induction results from mRNA stabilization and depends on its rapid turnover prior to stabilization. Exon 3 coding sequences were found to be both necessary and sufficient for cycloheximide inducibility of *myc* mRNA, suggesting that they determine translation-dependent instability of c-*myc* mRNA (46). Analyses of comparative steady-state mRNA levels also demonstrated that exon 3 coding sequences can act as an instability determinant under certain conditions. While they were dispensable for *myc* mRNA instability under growth conditions, their fusion to globin mRNA accelerated turnover of the fusion mRNA (47). Lavenu et al. also have suggested that exon 3 coding sequences are important determinants of posttranscriptional regulation of c-*myc* mRNA. Based on transgenic mouse studies, they believe that these sequences are responsible for tissue-specific patterns of c-*myc* mRNA expression as well as induction of c-*myc* mRNA in regenerating liver and after cycloheximide administration (32). Their results suggest that these sequences have a destabilizing effect that can be regulated under growth conditions, e.g., during liver regeneration, to allow higher levels of expression. Similarly, our data suggest that exon 3 coding sequences contain a conditional instability determinant that functions during C2C12 differentiation and mediates *myc* mRNA downregulation by destabilizing the mRNA.

Exon 2 sequences also target *myc* mRNA for downregulation, presumably by destabilizing the mRNA during differentiation. Previous studies examining cycloheximide inducibility of *myc* mRNA (46), mRNA decay rates after inhibition of transcription with actinomycin D (27), and analyses of comparative steady-state mRNA levels (47) failed to reveal the presence of a destabilizing element in exon 2. However, results of studies using global inhibitors of transcription or translation may be confounded by artifact, as has been demonstrated in studies of c-*fos* (43) and c-*myc* (46) mRNA metabolism. Previous comparisons of steady-state levels of *myc* or globin-*myc* fusion mRNAs deleting or fusing exon 2 sequences, which failed to reveal a destabilizing effect of these sequences, were carried out only under growth conditions (47). Results of studies presented here suggest that exon 2 sequences contain an instability determinant that is operative only under special conditions of cell or tissue differentiation. Interestingly, exon 2 sequences were previously implicated in the posttranscriptional regulation of c-*myc* mRNA by studies demonstrating that they determined tissue-specific expression of c-*myc* mRNA and inducibility of c-*myc* mRNA during liver regeneration and cycloheximide treatment (32) through a mechanism that did not depend on their translation (40). Our results suggest that *myc* exon 2 coding sequences also play an important role in the posttranscriptional downregulation of c-*myc* mRNA during myogenesis. In contrast to what was found in the liver, however, the ability of *myc* exon 2 (or exon 3) sequences to confer downregulation on globin mRNA in differentiating myoblasts requires their translation (unpublished observations).

In conclusion, we have examined the posttranscriptional regulation of mRNAs encoded by mutant *myc* and chimeric globin-*myc* and CAT-*myc* fusion genes to determine the *cis*-acting elements targeting c-*myc* mRNA for downregulation during C2C12 differentiation. Our data demonstrate that two elements, one in *myc* exon 2 and the other in the protein coding domain of *myc* exon 3, are both necessary for *myc* mRNA downregulation. While neither element alone is sufficient for downregulating *myc* or CAT-*myc* fusion mRNAs, either alone can confer downregulation when fused to β-globin mRNA. Finally, we demonstrate that mRNA downregulation does not result from altered nuclear processing but occurs as a result of postnuclear accelerated mRNA turnover.

ACKNOWLEDGMENTS

We thank Stephen Liebhaber for providing the β-globin cDNA clone and Xinkang Wang for providing rpL32. We also thank Eric Russell for critical reading of the manuscript and Muhammad Rehman for technical assistance.

This work was supported by National Research Service award GM16261-01 (N.M.Y.), Clinical Investigator award AR01956 (N.M.Y.), and Public Health Service grant GM47147 (W.M.F.L.) from the National Institutes of Health, by the McCabe Fund (N.M.Y.), and by American Cancer Society Faculty Research award FRA-406 (W.M.F.L.).

REFERENCES

1. **Armelin, H. A., M. C. Armelin, K. Kelly, T. Stewart, P. Leder, B. H. Cochran, and C. D. Stiles.** 1984. Functional role for c-myc in mitogenic response to

platelet-derived growth factor. Nature **310:**655–660.
2. **Bello-Fernandez, C., and J. L. Cleveland.** 1992. c-myc transactivates the ornithine decarboxylase gene. Curr. Top. Microbiol. Immunol. **182:**445–452.
3. **Bello-Fernandez, C., G. Packham, and J. L. Cleveland.** 1993. The ornithine decarboxylase gene is a transcriptional target of c-Myc. Proc. Natl. Acad. Sci. USA **90:**7804–7808.
4. **Bentley, D. L., and M. Groudine.** 1986. A block to elongation is largely responsible for decreased transcription of c-myc in differentiated HL60 cells. Nature **321:**702–706.
5. **Bernstein, P. L., D. J. Herrick, R. D. Prokipcak, and J. Ross.** 1992. Control of c-*myc* mRNA half-life in vitro by a protein capable of binding to a coding region stability determinant. Genes Dev. **6:**642–654.
6. **Blanchard, J. M., M. Piechaczyk, C. Dani, J. C. Chambard, A. Franchi, J. Pouyssegur, and P. Jeanteur.** 1985. c-myc gene is transcribed at high rate in G0-arrested fibroblasts and is post-transcriptionally regulated in response to growth factors. Nature **317:**443–445.
7. **Blau, H. M., G. K. Pavlath, E. C. Hardeman, C.-P. Chiu, L. Silberstein, S. G. Webster, S. C. Miller, and C. Webster.** 1985. Plasticity of the differentiated state. Science **230:**758–766.
8. **Casey, J. L., D. M. Koeller, V. C. Ramin, R. D. Klausner, and J. B. Harford.** 1989. Iron regulation of transferrin receptor mRNA levels requires iron-responsive elements and a rapid turnover determinant in the 3′ untranslated region of the mRNA. EMBO J. **8:**3693–3699.
9. **Cesarman, E., R. Dalla-Favera, D. Bentley, and M. Groudine.** 1987. Mutations in the first exon are associated with altered transcription of c-myc in Burkitt lymphoma. Science **238:**1272–1275.
10. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:**1991–1995.
11. **Coppola, J. A., and M. D. Cole.** 1986. Constitutive c-myc oncogene expression blocks mouse erythroleukaemia cell differentiation but not commitment. Nature **320:**760–763.
12. **Dani, C., J. M. Blanchard, M. Piechaczyk, S. El Sabouty, L. Marty, and P. Jeanteur.** 1984. Extreme instability of myc mRNA in normal and transformed human cells. Proc. Natl. Acad. Sci. USA **81:**7046–7050.
13. **Dani, C., N. Mechti, M. Piechaczyk, B. Lebleu, P. Jeanteur, and J. M. Blanchard.** 1985. Increased rate of degradation of c-myc mRNA in interferon-treated Daudi cells. Proc. Natl. Acad. Sci. USA **82:**4896–4899.
14. **Dean, M., R. A. Levine, and J. Campisi.** 1986. c-*myc* regulation during retinoic acid-induced differentiation of F9 cells is posttranscriptional and associated with growth arrest. Mol. Cell. Biol. **6:**518–524.
15. **Dony, C., M. Kessel, and P. Gruss.** 1985. Post-transcriptional control of *myc* and *p53* expression during differentiation of the embryonal carcinoma cell line F9. Nature **317:**637–639.
16. **Dudov, K. P., and R. P. Perry.** 1984. The gene family encoding the mouse ribosomal protein L32 contains a uniquely expressed intron-containing gene and an unmutated processed gene. Cell **37:**457–458.
17. **Eilers, M., S. Schirm, and J. M. Bishop.** 1991. The MYC protein activates transcription of the alpha-prothymosin gene. EMBO J. **10:**133–141.
18. **Freytag, S. O.** 1988. Enforced expression of the c-*myc* oncogene inhibits cell differentiation by precluding entry into a distinct predifferentiation state in G_0/G_1. Mol. Cell. Biol. **8:**1614–1624.
19. **Galaktionov, K., X. Chen, and D. Beach.** 1996. Cdc25 cell-cycle phosphatase as a target of c-myc. Nature **382:**511–517.
20. **Graves, R. A., N. B. Pandey, N. Chodchoy, and W. F. Marzluff.** 1987. Translation is required for regulation of histone mRNA degradation. Cell **48:**615–626.
21. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature **311:**433–438.
22. **Griep, A. E., and H. Westphal.** 1988. Antisense Myc sequences induce differentiation of F9 cells. Proc. Natl. Acad. Sci. USA **85:**6806–6810.
23. **Grosso, L. E., and H. C. Pitot.** 1985. Transcriptional regulation of c-myc during chemically induced differentiation of HL-60 cultures. Cancer Res. **45:**847–850.
24. **Heikkila, R., G. Schwab, E. Wickstrom, S. L. Loke, D. H. Pluznik, R. Watt, and L. M. Neckers.** 1987. A c-myc antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G0 to G1. Nature **328:**445–449.
25. **Henriksson, M., and B. Luscher.** 1996. Proteins of the Myc network: essential regulators of cell growth and differentiation. Adv. Cancer Res. **68:**109–182. (Review.)
26. **Herrick, D. J., and J. Ross.** 1994. The half-life of c-*myc* mRNA in growing and serum-stimulated cells: influence of the coding and 3′ untranslated regions and role of ribosome translocation. Mol. Cell. Biol. **14:**2119–2128.
27. **Jones, T. R., and M. D. Cole.** 1987. Rapid cytoplasmic turnover of c-*myc* mRNA: requirement of 3′ untranslated sequences. Mol. Cell. Biol. **7:**4513–4521.
28. **Kaczmarek, L., J. K. Hyland, R. Watt, M. Rosenberg, and R. Baserga.** 1985. Microinjected c-myc as a competence factor. Science **228:**1313–1315.
29. **Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder.** 1983. Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell **35:**603–610.
30. **Knight, E. Jr., E. D. Anton, D. Fahey, B. K. Friedland, and G. J. Jonak.** 1985. Interferon regulates c-*myc* gene expression in Daudi cells at the post-transcriptional level. Proc. Natl. Acad. Sci. USA **82:**1151–1154.
31. **Krystal, G., M. Birrer, J. Way, M. Nau, E. Sausville, C. Thompson, J. Minna, and J. Battey.** 1988. Multiple mechanisms for transcriptional regulation of the *myc* gene family in small-cell lung cancer. Mol. Cell. Biol. **8:**3373–3381.
32. **Lavenu, A., S. Pistoi, S. Pournin, C. Babinet, and D. Morello.** 1995. Both coding exons of the c-*myc* gene contribute to its posttranscriptional regulation in the quiescent liver and regenerating liver and after protein synthesis inhibition. Mol. Cell. Biol. **15:**4410–4419.
33. **Levine, R. A., J. E. McCormack, A. Buckler, and G. E. Sonenshein.** 1986. Transcriptional and posttranscriptional control of c-*myc* gene expression in WEHI 231 cells. Mol. Cell. Biol. **6:**4112–4116.
34. **Makino, R., K. Akiyama, J. Yasuda, S. Mashiyama, S. Honda, T. Sekiya, and K. Hayashi.** 1994. Cloning and characterization of a c-myc intron binding protein (MIBP1). Nucleic Acids Res. **22:**5679–5685.
35. **McMaster, G. K., and G. C. Carmichael.** 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA **74:**4835–4838.
36. **Mechti, N., M. Piechaczyk, J. M. Blanchard, L. Marty, A. Bonnieu, P. Jeanteur, and B. Lebleu.** 1986. Transcriptional and post-transcriptional regulation of c-myc expression during the differentiation of murine erythroleukemia Friend cells. Nucleic Acids Res. **14:**9653–9666.
37. **Mullner, E. W., and L. C. Kuhn.** 1988. A stem-loop in the 3′ untranslated region mediates iron-dependent regulation of transferrin receptor mRNA in the cytoplasm. Cell **53:**815–825.
38. **Nepveu, A., and K. B. Marcu.** 1986. Intragenic pausing and anti-sense transcription within the murine c-myc locus. EMBO J. **5:**2859–2865.
39. **Philipp, A., A. Schneider, I. Vasrik, K. Finke, Y. Xiong, D. Beach, K. Alitalo, and M. Eilers.** 1994. Repression of cyclin D1: a novel function of MYC. Mol. Cell. Biol. **14:**4032–4043.
40. **Pistoi, S., J. Roland, C. Babinet, and D. Morello.** 1996. Exon 2-mediated c-*myc* mRNA decay in vivo is independent of its translation. Mol. Cell. Biol. **16:**5107–5116.
41. **Prochownik, E. V., J. Kukowska, and C. Rodgers.** 1988. c-*myc* antisense transcripts accelerate differentiation and inhibit G1 progression in murine erythroleukemia cells. Mol. Cell. Biol. **8:**3683–3695.
42. **Samaniego, F., J. Chin, K. Iwai, T. A. Rouault, and R. D. Klausner.** 1994. Molecular characterization of a second iron-responsive element binding protein, iron regulatory protein 2. Structure, function, and post-translational regulation. J. Biol. Chem. **269:**30904–30910.
43. **Schiavi, S. C., C. L. Wellington, A. B. Shyu, C. Y. Chen, M. E. Greenberg, and J. G. Belasco.** 1994. Multiple elements in the c-fos protein-coding region facilitate mRNA deadenylation and decay by a mechanism coupled to translation. J. Biol. Chem. **269:**3441–3448.
44. **Stone, J., T. de Lange, G. Ramsay, E. Jakobovits, J. M. Bishop, H. Varmus, and W. Lee.** 1987. Definition of regions in human c-*myc* that are involved in transformation and nuclear localization. Mol. Cell. Biol. **7:**1697–1709.
45. **Wisdom, R., and W. Lee.** 1990. Translation of c-*myc* mRNA is required for its post-transcriptional regulation during myogenesis. J. Biol. Chem. **265:**19015–19021.
46. **Wisdom, R., and W. Lee.** 1991. The protein-coding region of c-*myc* mRNA contains a sequence that specifies rapid mRNA turnover and induction by protein synthesis inhibitors. Genes Dev. **5:**232–243.
47. **Yeilding, N. M., M. T. Rehman, and W. M. Lee.** 1996. Identification of sequences in c-*myc* mRNA that regulate its steady-state levels. Mol. Cell. Biol. **16:**3511–3522.
48. **Yen, T. J., P. S. Machlin, and D. W. Cleveland.** 1988. Autoregulated instability of beta-tubulin mRNAs by recognition of the nascent amino terminus of beta-tubulin. Nature **334:**580–585.
49. **Yokoyama, K., and F. Imamoto.** 1987. Transcriptional control of the endogenous MYC protooncogene by antisense RNA. Proc. Natl. Acad. Sci. USA **84:**7363–7367.