MOLECULAR AND CELLULAR BIOLOGY, May 1986, p. 1412–1421
0270-7306/86/0501412-10$02.00/0

Vol. 6, No. 5

Transcriptional and Posttranscriptional Control of c-*myc* during Myogenesis: Its mRNA Remains Inducible in Differentiated Cells and Does Not Suppress the Differentiated Phenotype

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Received 18 December 1985/Accepted 18 February 1986

It is widely accepted that the cellular oncogene c-*myc* plays an important role in the control of cell proliferation and that its expression diminishes in differentiated cells. We examined whether there is a correlation between c-*myc* expression and cell proliferation or differentiation by using a subclone of a rat skeletal muscle cell line L_6E_9. Myoblasts irreversibly withdraw from the cell cycle, fuse to form multinucleated myotubes, and express muscle-specific genes (terminal differentiation). Muscle-specific genes can also be expressed in the absence of fusion (biochemical differentiation). Such mononucleated but biochemically differentiated cells can be stimulated to reenter the cell cycle. c-*myc* was induced by insulin, insulin-like growth factor, or serum factors in G_0-arrested cells, whereas induction by protein synthesis inhibitors or superinduction by protein synthesis inhibitors in combination with serum factors occurred in all physiological states tested. We found that c-*myc* expression was reduced in biochemically and terminally differentiated cells as well as in quiescent undifferentiated cells but that it remained inducible by growth factors in all three physiological states. Results of nuclear runoff transcription assays suggested that the induction of c-*myc* mRNA by growth factors and its deinduction in these physiological states were regulated mainly at the transcriptional level. In contrast, induction and superinduction of c-*myc* mRNA by protein synthesis inhibitors alone and in combination with growth factors, respectively, were regulated posttranscriptionally mainly by stabilization of c-*myc* mRNA. Moreover, c-*myc* and muscle-specific genes could be simultaneously transcribed in both biochemically and terminally differentiated cells. These results indicate that irreversible repression of c-*myc* is not required for terminal myogenic differentiation and that its expression is insufficient by itself to suppress the differentiated phenotype.

The levels of several cellular oncogene mRNAs and proteins have been shown to fluctuate, depending on the state of cell proliferation and differentiation (22, 23, 29, 55, 61, 62). Among these oncogenes, c-*myc* has been studied most extensively. This cellular oncogene is rapidly induced in quiescent fibroblasts or lymphocytes stimulated to proliferate by growth factors or mitogens (23, 29, 43) and in rat liver after hepatectomy (37). In addition, transfected human and mouse c-*myc* genes or microinjected c-*myc* protein stimulates DNA synthesis and the proliferation of fibroblast cell lines in platelet-derived growth factor (PDGF)-free medium (1, 28). These phenomena suggest that c-*myc* plays an important role in the control of cell proliferation. Furthermore, during terminal differentiation of a variety of cell lines, the level of c-*myc* mRNA decreases markedly (6, 11, 14, 22, 31, 54, 62). These observations raise the possibility that c-*myc* deinduction is involved in the irreversible withdrawal of terminally differentiated cells from the cell cycle. Despite the attractiveness of this hypothesis, however, it had not been previously determined whether the down-regulation of c-*myc* is reversible or irreversible in terminally differentiated cells, whether it is a requisite for the induction or maintenance of the differentiated phenotype, or whether it is the cause or the consequence of the reduced cell growth rate that generally leads to the differentiated state. Myogenic cells are a convenient model system to address some of these questions.

Myoblast cells grown in the presence of enough growth factors remain in a proliferative state and do not express muscle-specific genes (for a review, see reference 44). When the concentration of growth factors is reduced, the cells cease DNA synthesis, irreversibly withdraw from the cell cycle (commitment), and fuse to form multinucleated myotubes (terminal differentiation) (44, 48, 63). Concurrent with these cellular events, a battery of myofibrillar genes including myosin heavy chain (MHC), myosin light chains (MLCs), α-actin, and α- and β-tropomyosin, as well as troponin T, I, and C are induced, whereas other genes such as β- and γ-actin are deinduced (3, 7, 21, 26, 40, 42, 57). In addition, the muscle-specific contractile protein genes can be induced in the absence of cell fusion (biochemical differentiation) through manipulation of the Ca^{2+} concentration in the culture medium of wild-type cells (15, 46) and the use of cell lines that are temperature sensitive for the irreversible withdrawal from the cell cycle and cell fusion steps (47). These mononucleated but biochemically differentiated cells can be stimulated to reenter the cell cycle in the presence of high concentrations of growth factors with the concomitant and rapid deinduction of muscle-specific gene expression (12, 13, 45, 47). Therefore, in these systems cell proliferation and acquisition of differentiated phenotypes appear to be mutually exclusive phenomena (47). To permanently induce the expression of muscle-specific genes, it is necessary for the cells to irreversibly withdraw from the cell cycle and to become unresponsive to growth factor stimulation for DNA replication. Since the biochemical mechanisms involved in this process are presently not known, it is of interest to determine whether increased levels of c-*myc* in response to

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growth stimulation could be involved in the reinitiation of cell proliferation and concomitant deinduction of muscle-specific genes in uncommitted but biochemically differentiated cells.

In this study we determined whether there is a cause-effect relationship between c-*myc* gene expression and induction or deinduction of the differentiated myogenic phenotypes by using the rat L_6E_9 myogenic cell line. We found that c-*myc* expression was reduced in biochemically and terminally differentiated cells as well as in quiescent undifferentiated cells but that it remained inducible by growth factors in all three physiological states. Results of nuclear runoff transcription assays suggested that the induction of c-*myc* mRNA by growth factors and its deinduction in these physiological states are regulated mainly at the transcriptional level. In contrast, induction and superinduction of c-*myc* mRNA by protein synthesis inhibitors alone and in combination with growth factors, respectively, were regulated posttranscriptionally mainly by stabilization of c-*myc* mRNA. Moreover, c-*myc* and muscle-specific genes could be simultaneously transcribed in both biochemically and terminally differentiated cells. These results indicate that irreversible repression of c-*myc* is not required for terminal differentiation and that its expression is not sufficient by itself to suppress the differentiated phenotype.

MATERIALS AND METHODS

Cell lines and culture conditions. L_6E_9-B cells were obtained by recloning the rat myogenic cell line L_6E_9 (44) in 96-well plates. This cell line was maintained as proliferating myoblasts in Dulbecco modified Eagle (DME) medium (GIBCO Laboratories) supplemented with 20% fetal calf serum (FCS; GIBCO) (growth medium). The generation time of L_6E_9-B cells, 16 h, is similar to that of L_6E_9 cells, but they differentiate faster than the parental cell line. To induce terminal differentiation, cells were either transferred from growth medium to DME medium supplemented with 5% horse serum (GIBCO) (differentiation medium) or replated in differentiation medium. These cells start fusion on day 2 and about 90% of the nuclei are in myotubes by day 3. MHC, α-actin, troponin T, and MLC2 mRNAs were detected 33 h after the cells were transferred into differentiation medium and reached maximum levels by 60 h (see Fig. 3A). The cells were incubated at 37°C under a humidified atmosphere containing 7.5% CO_2. L_6E_9-E cells were obtained by recloning the L_6E_9 cells infected with Moloney murine sarcoma virus *ts*110 (27) in 96-well plates. The cells were maintained in DME medium supplemented with 10% FCS at 33°C under 7.5% CO_2.

The sources of growth factors used in this study were as follows: insulin was from Sigma Chemical Co.; insulin-like growth factor I (IGF-I, somatomedin C) was from AMGen Biologicals; fibroblast growth factor (FGF), PDGF, and epidermal growth factor (EGF) were from Collaborative Research, Inc.

Isolation of cytoplasmic RNA and RNA blotting. Cytoplasmic RNA was isolated by a modification of the method of Favaloro et al. (18), with 10 mM vanadyl ribonucleoside complexes in lysis buffer (38). Samples (10 μg) of each cytoplasmic RNA were size fractionated and transferred to nitrocellulose paper as described previously (47, 60). Nick-translated ^{32}P-labeled muscle-specific cDNA plasmids pMHC25 (41), pAc269 (56), pTnT15, and pMLC2-18 (21) or a 1.7-kilobase (kb) *Eco*RI-*Bam*HI fragment of human c-*myc* plasmid pMC41-3RC (9) were hybridized to the filters as described previously (47).

Nuclear runoff transcription assay. Nuclei were isolated and stored at −80°C as described by Groudine et al. (24) and Greenberg and Ziff (23). Nuclear runoff transcription was performed by the method of Greenberg and Ziff (23). Samples of an *Eco*RI-*Bam*HI fragment of human c-*myc* plasmid pMC41-3RC (9), a *Pst*I-*Eco*RI fragment of chicken actin plasmid pAc269 (56), an *Eco*RI-restricted rat embryonic MHC genomic fragment (49), and a *Pst*I-restricted rat preproinsulin II plasmid (36) were dot spotted onto nitrocellulose filters (23) by using a minifold apparatus (Schleicher & Schuell, Inc.). Approximately 2 μg of DNA was applied per well.

[^{3}H] thymidine labeling and autoradiography. Cells grown on glass cover slips were incubated for 24 h with 0.3 μCi of [6-^{3}H]thymidine (TdR) (specific activity, 20.0 Ci/mmol; Amersham Corp.) per ml in an appropriate medium and processed for autoradiography as described by Stein and Yanishevsky (58), with Kodak nuclear track emulsion type NTB2. Specimens were exposed at 4°C for 5 days, developed with Kodak D19 developer, and stained with Giemsa staining solution.

RESULTS

Serum, insulin, and insulin-like growth factor but not PDGF, FGF, or EGF induce c-*myc* in quiescent myogenic cells. c-*myc* mRNA is induced in quiescent BALB/c-3T3 cells during the transition from G_0 to G_1 phase by PDGF or serum (6, 29). FGF and a tumor promoter, 12-*O*-tetradecanoyl phorbol 13-acetate, substitute to some extent for PDGF in the induction of c-*myc* mRNA, but EGF or insulin alone has virtually no effect (29). To examine the pattern of c-*myc* induction by growth factors in L_6E_9-B cells, cultures of logarithmically growing and quiescent cells were transferred to fresh growth medium containing 20% FCS. The level of cytoplasmic c-*myc* mRNA at different times was determined by hybridization of a c-*myc* DNA probe (9). The level of c-*myc* mRNA was low in both proliferating and quiescent cells (Fig. 1A and C, time 0). Serum stimulation had little effect on the level of c-*myc* mRNA in proliferating cultures (Fig. 1A) but caused a rapid induction within 1 h in quiescent cells (Fig. 1C), with a return to the basal level after 8 h.

To identify serum factors responsible for c-*myc* mRNA induction, quiescent L_6E_9-B cells were transferred to DME medium supplemented with 1% HS and individual purified growth factors. In these cells, 1% horse serum did not change the level of c-*myc* mRNA (Fig. 2). L_6E_9-E cells, which express c-*myc* mRNA at high levels when grown at 33°C, were used as a reference for the c-*myc* mRNA level. Insulin (30 μg/ml) or IGF-I (1 μg/ml) induced c-*myc* mRNA to levels comparable to those induced by 20% FCS (Fig. 2). On the contrary, PDGF, FGF, and EGF did not induce c-*myc* at the concentrations of 5 U/ml, 0.2 μg/ml, and 0.1 μg/ml, respectively, which are severalfold higher than the concentrations needed to induce c-*myc* in a variety of cell types (5, 29, 43). In L_6E_9-B cells, therefore, c-*myc* induction by serum is at least in part mediated by IGF-I and insulin but not by EGF, FGF, or PDGF. In light of these results, it is interesting that high concentrations of insulin, IGF-I, and IGF-II stimulate the proliferation of L_6 myoblasts (16, 20; J. R. Florini, personal communication), the parental cell line of L_6E_9 (44), and L_6E_9-B cells. In contrast, neither FGF nor EGF causes proliferation of these cells (Florini, personal communication). This correlation between stimulation of

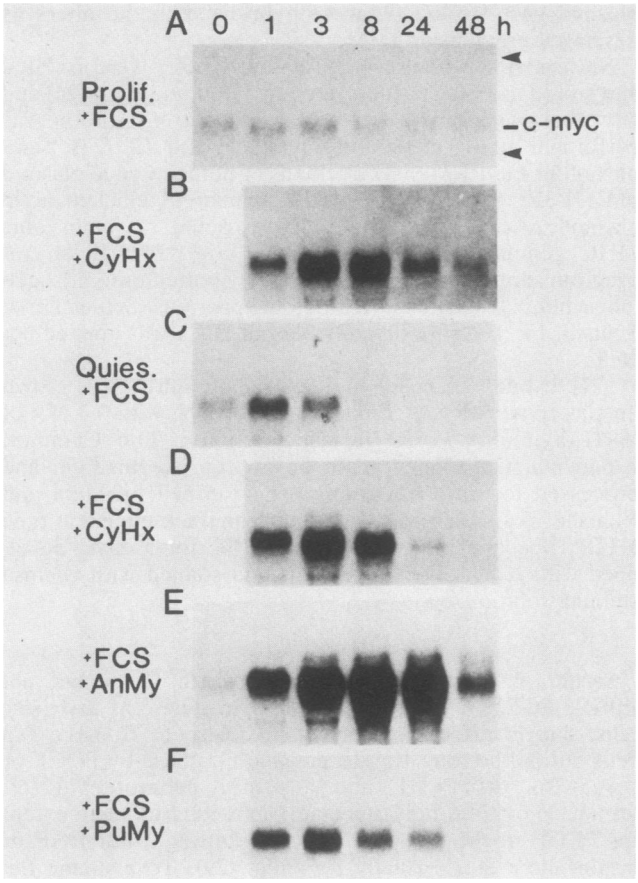

FIG. 1. Induction and superinduction of c-*myc* mRNA by FCS and protein synthesis inhibitors, repsectively. (A and B) Proliferating L_6E_9-B cells maintained for 3 days in growth medium (~99% of the cells synthesized DNA in 24 h) were transferred to fresh growth medium (A) or to growth medium containing 10 μg of cycloheximide per ml (B). (C through F) Overconfluent and quiescent L_6E_9-B cells cultured for 5 days in growth medium (<10% of the cells synthesized DNA in 24 h) were transferred to fresh growth medium (C), growth medium containing 10 μg of cycloheximide per ml (D), growth medium containing 10 μg of anisomycin per ml (E), or growth medium containing 1 μg of puromycin per ml (F). Cytoplasmic RNA was isolated 1, 3, 8, 24, and 48 h later, and the c-*myc* mRNA level was analyzed by RNA blotting. The position of c-*myc* mRNA (2.4 kilobases) is indicated. Arrowheads refer to the positions of 28S and 18S rRNAs.

proliferation and c-*myc* induction in response to different growth factors is consistent with the hypothesis that c-*myc* induction is involved in myoblast proliferation, as has been postulated for other cell types. However, we cannot rule out the possibility that the activation of growth factor receptors by the binding of specific ligands induces c-*myc* mRNA and cell proliferation by independent mechanisms.

c-*myc* mRNA is superinduced by protein synthesis inhibitors in the presence of serum factors. In several cell types, c-*myc* mRNA is induced by a protein synthesis inhibitor, cycloheximide, and superinduced by cycloheximide in combination with growth factors (29, 43) on with liver regeneration (37). These findings have suggested the participation of a labile protein as a repressor of c-*myc* gene expression at the transcriptional level (32). However, recent work indicates that, in several cell types, posttranscriptional events affecting c-*myc* mRNA stability play a fundamental role in regulating the cytoplasmic c-*myc* mRNA levels under a variety of conditions and in response to cycloheximide (4, 10, 14, 30, 51).

When cycloheximide (10 μg/ml) was applied to proliferating and quiescent L_6E_9-B cells in conjunction with fresh growth medium, c-*myc* mRNA was superinduced in both cultures (Fig. 1B and D), and reached similar maximal levels in 8 and 3 h, respectively. This effect seems to be due to protein synthesis inhibition and not to secondary effects of the drug because other protein synthesis inhibitors with different mechanisms of action, such as anisomycin and puromycin (50) also superinduced c-*myc* mRNA in quiescent cells when used in addition to serum (Fig. 1E and F). Anisomycin was more effective than cycloheximide, whereas puromycin was less effective in terms of the level of superinduction. These results suggest that a short-lived protein is involved in the negative regulation of c-*myc* mRNA but do not address the question of whether this regulation occurs at the transcriptional or posttranscriptional level (see below).

c-*myc* expression changes biphasically during biochemical and terminal myogenic differentiation. In a number of differentiating cell systems, the level of c-*myc* mRNA decreases in response to inducers of differentiation before the acquisition of the differentiated phenotype (6, 11, 14, 22, 31, 54, 62). To test whether changes in c-*myc* gene expression are involved in the commitment step leading to irreversible differentiation of myogenic cells, we examined c-*myc* mRNA levels in L_6E_9-B cells at different times during biochemical and terminal differentiation. As probes for the differentiated state, we used cDNA clones specific for the components of the thick (MHC and MLC2) and thin (actin and troponin T) filaments (21, 41, 56). Muscle-specific MHC, α-actin, troponin T, and MLC2 mRNAs appeared 36 h after switching to differentiation medium and reached maximal levels 72 h later, when about 90% of the nuclei were already into myotubes (terminal differentiation) (Fig. 3A). c-*myc* mRNA levels decreased four- to fivefold between 3 and 12 h after switching to differentiation medium. This mRNA, however, was transiently restored to almost basal levels between 18 and 36 h and declined again in parallel with the accumulation of muscle-specific mRNAs (Fig. 3B).

L_6E_9-B cells induced to differentiate in the presence of

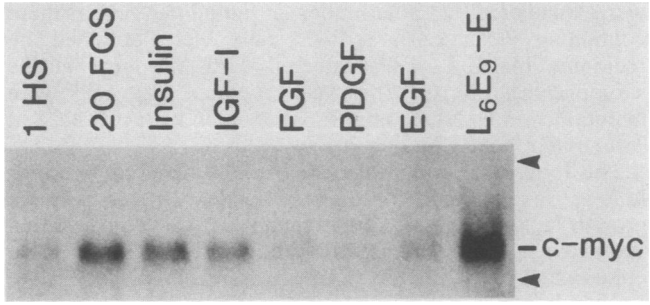

FIG. 2. Insulin and IGF-I induce c-*myc* mRNA. Quiescent L_6E_9-B cells maintained for 5 days in growth medium were transferred to DME medium containing 1% horse serum (1 HS), 20% FCS (20 FCS), or 1% horse serum and a single species of the following growth factors: 30 μg of insulin per ml, 1 μg of IGF-1 per ml, 0.2 μg of FGF per ml, 5 U of PDGF per ml, or 0.1 μg of EGF per ml. Cytoplasmic RNA was isolated 3 h later from each culture, and the c-*myc* mRNA level was analyzed by RNA blotting. L_6E_9-E cell RNA was used for a reference of c-*myc* mRNA level. Arrowheads indicate the positions of 28S and 18S rRNAs.

1.75 mM EGTA express muscle-specific mRNAs but neither fuse nor irreversibly withdraw from the cell cycle (biochemical differentiation) (15, 46). The expression of both muscle-specific and c-*myc* mRNAs under these conditions (Fig. 3C and D) was very similar to that exhibited by cells in the process of terminal differentiation (Fig. 3A and B). The only difference noted is that during biochemical differentiation the expression of the muscle-specific genes and the secondary expression of c-*myc* were slightly delayed. These results argue against, but do not exclude, a direct cause-effect relationship between c-*myc* deinduction and the commitment to terminal differentiation with irreversible withdrawal from the cell cycle. However, the timing of c-*myc* down-regulation and the induction of muscle-specific genes in both terminally and biochemically differentiating cells raise the possibility that these two phenomena are sequential steps in the same differentiation pathway.

The biphasic expression of c-*myc* mRNA is also observed during terminal differentiation of mouse erythroleukemia cells (31), where the basal level and secondary expression are higher than those in L_6E_9-B cells. It has been postulated that a positively acting protein factor transiently expressed during differentiation is responsible for the secondary expression of c-*myc* in erythroleukemia cells (31). To test this hypothesis in L_6E_9-B cells, cycloheximide was added to parallel cultures at 6-h intervals for 3 days during terminal differentiation. Cytoplasmic mRNA was isolated 3 h after the addition of cycloheximide and analyzed for c-*myc* mRNA levels. c-*myc* mRNA was induced to high and similar levels throughout the experiment, independently of the differentiated state of the cells and the c-*myc* mRNA level in the untreated cultures (Fig. 3E). These results argue against the presence of a newly synthesized positive regulatory protein being responsible for the secondary expression of c-*myc*. On the contrary, they suggest that the biphasic modulation of c-*myc* mRNA levels during L_6E_9-B cell differentiation is negatively regulated by short-lived factor(s) sensitive to protein synthesis inhibitors.

c-*myc* mRNA can be induced and superinduced in biochemically and terminally differentiated L_6E_9-B cells. Cell proliferation and maintenance of the differentiated phenotype are mutually exclusive phenomena in myogenic cells (47) as well as in other differentiating cells (25). Since c-*myc* has been postulated to play an important role in the control of cell proliferation, and its expression decreases in differentiated cells, irreversible deinduction of c-*myc* could be one of the events leading to terminal differentiation, whereas its reversible down-regulation could provide the basis for modulation of the differentiated phenotype of cells able to reenter the cell cycle.

To test the above hypothesis, the inducibility of c-*myc* mRNA in response to serum was determined in biochemically and terminally differentiated L_6E_9-B cells. Biochemically differentiated cells cultured for 4 days in the presence of 1.75 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] were switched to medium containing 20% FCS–EGTA. c-*myc* mRNA was induced at high levels within 1 h and reached maximal induction in 3 h (Fig. 4B). This result is not surprising, given that the majority of these biochemically differentiated cells are able to reenter the cell cycle in response to serum stimulation (12; see below). Interestingly, however, the levels of muscle-specific mRNAs were not greatly affected by these manipulations and remained high even 96 h after serum stimulation, with the exception of a transient decrease in the MHC mRNA level (Fig. 4A). These results are in apparent contradiction to previous reports that serum stimulation produces a rapid deinduction of muscle-specific mRNAs (46, 47). This discrepancy is due to different protocols for serum stimulation. When EGTA-treated biochemically differentiated L_6E_9-B cells were replated in fresh growth medium, a rapid deinduc-

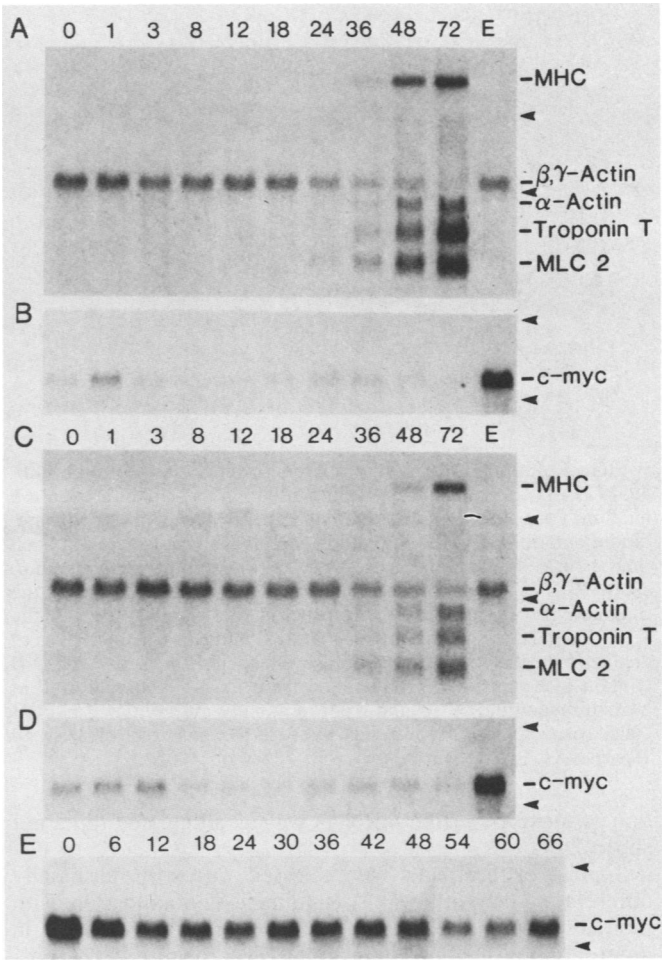

FIG. 3. Expression of muscle-specific and c-*myc* mRNAs during terminal and biochemical differentiation. (A and B) L_6E_9-B cells were plated at the density of 4×10^6 cells per 150-mm dish in growth medium, maintained for 24 h, and then transferred to differentiation medium. At each time (hours) shown in the figure after the switch to differentiation medium, cytoplasmic RNA was isolated, and the c-*myc* mRNA level was analyzed by Northern blotting (B). After erasing the radioactivity by washing the blot for 30 min in 0.01× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 90°C, the levels of muscle-specific (MHC, α-actin, troponin T, and MLC2) and β- and γ-actin mRNAs were analyzed by hybridization of the probes to the same nitrocellulose filter (A). (C and D) L_6E_9-B cells were plated and maintained by the methods used for those in panels A and B and then transferred to differentiation medium containing 1.75 mM EGTA. Cytoplasmic RNA was isolated, and the levels of c-*myc* mRNA (D) and muscle-specific and β- and γ-actin mRNAs (C) were analyzed by the methods used for panels B and A, respectively. Lane E is cytoplasmic RNA isolated from L_6E_9-E cells cultured in growth medium at 33°C. (E) L_6E_9-B cells cultured for 24 h in growth medium were transferred to differentiation medium, and then cycloheximide was added at 6-h intervals to parallel cultures to a final concentration of 10 μg/ml. Three hours after the administration of the drug, cytoplasmic RNA was isolated from each culture, and c-*myc* mRNA levels were analyzed as above. Arrowheads indicate the positions of 28S and 18S rRNAs.

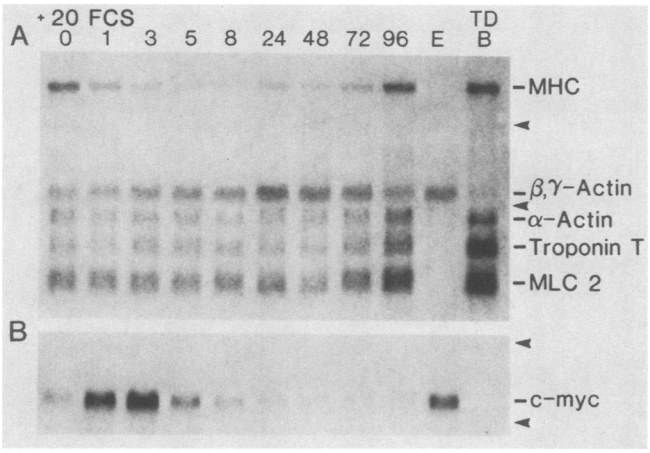

FIG. 4. Induction of c-*myc* mRNA in biochemically differentiated L_6E_9-B cells. Cells were maintained for 4 days in differentiation medium containin 1.75 mM EGTA and transferred to growth medium containing 20% FCS. Cytoplasmic RNA was isolated at each time (hours) shown in the figure after the transfer to growth medium. c-*myc* mRNA level was analyzed by RNA blotting (B). After the radioactivity was erased as described in the legend to Fig. 3, the levels of muscle-specific mRNAs and β- and γ-actin mRNA were analyzed on the same nitrocellulose filter (A). Lanes E and TD-B contain cytoplasmic RNAs isolated from L_6E_9-E cells cultured in growth medium at 33°C and from terminally differentiated L_6E_9-B cells, respectively. Arrowheads indicate the positions of 28S and 18S rRNAs.

tion of muscle-specific mRNAs was also detected (data not shown).

Similar experiments were carried out with terminally differentiated myotubes. To minimize contamination with mononucleated cells, cultures maintained for 5 days in differentiation medium (more than 95% fusion) were transferred to growth medium containing 10 μM 1-β-D-arabinofuranosylcytosine (19) for an additional 5 days. This treatment eliminates from the culture all the cells able to reenter the cell cycle and leaves a pure population of myotubes, as confirmed by microscopic observation, the high level of expression of the muscle-specific genes, and the complete disappearance of β- and γ-actin mRNA, a marker of undifferentiated cells (Fig. 5A). In this pure culture of terminally differentiated cells, c-*myc* mRNA was induced to a significant extent 1 h after serum stimulation and returned to the basal level 8 h later (Fig. 5B). This c-*myc* induction had no effect on the level of muscle-specific mRNAs detected in these cells. Moreover, by using immunofluorescence microscopy with MF20, a monoclonal antibody that recognizes the sarcomeric MHC (2) expressed in the L_6E_9 cell line (47), it was demonstrated that the expression of this protein is also not affected by the induction of c-*myc* (data not shown). When cycloheximide was added to the terminally differentiated cells in combination with serum, c-*myc* mRNA was superinduced and remained at high levels for more than 24 h (Fig. 5B). This result suggests that the low basal level of c-*myc* mRNA detected in terminally differentiated cells is negatively regulated by a mechanism similar to that operating in proliferating and in quiescent cells. The cycloheximide treatment did not markedly alter the level of muscle-specific mRNAs except that of MHC mRNA (Fig. 5A). The mechanism involved in this MHC mRNA induction is not presently understood and is likely to be different from c-*myc* induction, considering the long half-life of MHC mRNA (40; see below).

[^{3}H]TdR labeling, followed by autoradiography, was performed to determine the effect of serum stimulation and c-*myc* induction on DNA synthesis in biochemically and terminally differentiated cells. Biochemically differentiated cells cultured for 4 days in differentiation medium containing EGTA were incubated with [^{3}H]TdR for 24 h in the same medium. Less than 2% of the cells incorporated [^{3}H]TdR into the nuclear DNA (Fig. 6A). When biochemically differentiated cells were fed with fresh growth medium containing [^{3}H]TdR, more than 60% of them reinitiated DNA synthesis in the first 24 h (Fig. 6B), and up to 90% did so after 48 h (data not shown). In contrast, no DNA synthesis was detected in terminally differentiated myotubes even after serum stimulation (Fig. 6C).

These results demonstrate that c-*myc* mRNA remains inducible in both biochemically and terminally differentiated L_6E_9-B cells. They rule out the possibility that permanent repression of this gene is involved in the irreversible withdrawal from the cell cycle that myoblasts undergo in the process of terminal differentiation. Furthermore, they indicate that c-*myc* induction is insufficient by itself to trigger DNA synthesis and cell proliferation of terminally differentiated cells even in the presence of serum.

c-*myc* and muscle-specific genes are concomitantly transcribed. The results presented above indicate that high levels of c-*myc* and muscle-specific mRNAs can coexist in biochemically and terminally differentiated cells. This coexpression argues against the possibility that c-*myc* induction and down-regulation are directly involved in the repression and induction, respectively, of muscle-specific genes. However, because the half-life of c-*myc* mRNA is very short (15 to 60 min) (10, 39, 51), whereas that of muscle-specific mRNAs is long (55 to 60 h) (40; L. I. Garfinkel, Ph.D. thesis, Albert Einstein College of Medicine, New York, N.Y., 1982), it cannot be determined from the RNA blot analysis whether these genes are transcribed concurrently or whether

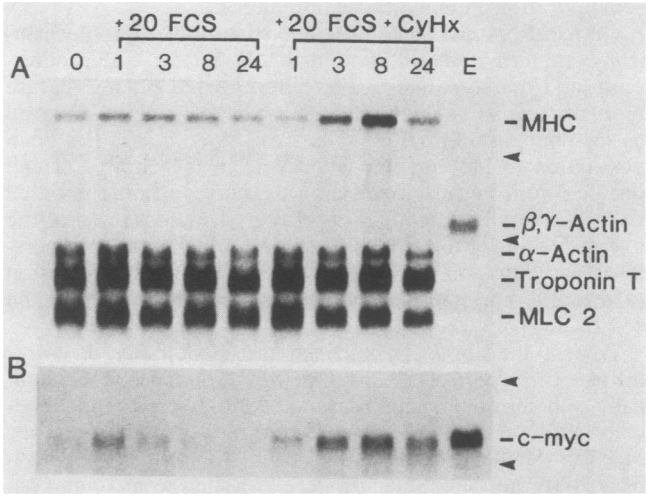

FIG. 5. Induction and superinduction of c-*myc* mRNA in terminally differentiated L_6E_9-B cells. Cultures of pure L_6E_9-B myotubes (see the text for details) were transferred to growth medium containing 20% FCS (+20 FCS) or the growth medium containing 10 μg of cycloheximide per ml (+20 FCS + CyHx), and 1, 3, 8, and 24 h later, cytoplasmic RNA was isolated from each culture. c-*myc* mRNA levels were analyzed by RNA blotting (B). After the radioactivity was erased as described for Fig. 3, the levels of muscle-specific mRNAs and β- and γ-actin mRNA were analyzed on the same nitrocellulose filter (A). E is as on Fig. 4. Arrowheads indicate the positions of 28S and 18S rRNAs.

induction of c-*myc* rapidly and transiently represses the transcription of muscle-specific genes. For this reason, nuclear runoff transcripts labeled with [^{32}P]UTP were isolated from biochemically differentiated L_6E_9-B cells maintained for 4 days in differentiation medium containing EGTA at the times shown in Fig. 7B after transfer to fresh growth medium. Equivalent amounts of incorporated radioactivity were hybridized to c-*myc* (9) and MHC (49) genomic fragments as well as to an α-actin cDNA, which also hybridizes to β- and γ-actin transcripts (56) immobilized to nitrocellulose filters. Nuclei isolated from biochemically differentiated cells (Fig. 7B, time 0) exhibited a concomitant transcription of c-*myc*, actin, and MHC genes. Stimulation with serum resulted in a rapid increase in c-*myc* and actin transcription within 30 min. Transcription of these two genes reached maximum levels 60 min after stimulation and remained at high levels 240 min later. The increased actin signal probably reflects the induction of the β- and γ-actin gene transcription in response to serum, as has been shown in other cell types (14, 23). In contrast, the level of MHC gene transcription remained constant throughout the experiment. That the signals detected in Fig. 7 are specific is supported by their complete inhibition by a concentration of α-amanitin sufficient to inhibit RNA polymerase II and the virtual absence of hybridization to a preproinsulin cDNA probe (36) which is not expressed in muscle cells (Fig. 7A). These results indicate that transcription of c-*myc* and muscle-specific genes can coexist. It is also clear that c-*myc* transcription is

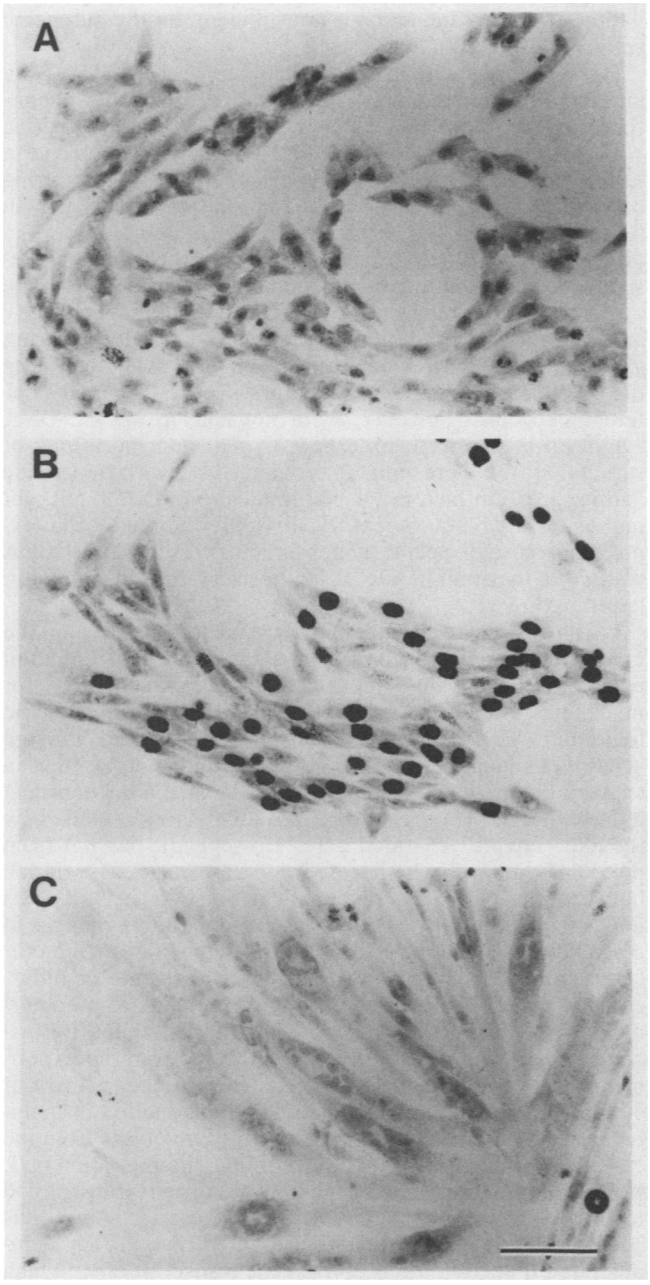

FIG. 6. Autoradiograms of biochemically and terminally differentiated L_6E_9-B cells labeled with [^{3}H]TdR. (A) L_6E_9-B cells were cultured on glass cover slips for 4 days in differentiation medium containing 1.75 mM EGTA and then incubated with 0.3 μCi of [^{3}H]TdR per ml for 24 h in the same medium. The cells were processed for autoradiography. (B) L_6E_9-B cells maintained for 4 days in differentiation medium containing 1.75 mM EGTA were transferred to growth medium containing [^{3}H]TdR and incubated for 24 h. (C) L_6E_9-B cells cultured for 7 days in differentiation medium were transferred to growth medium containing [^{3}H]TdR and incubated for 24 h. Bar, 100 μm.

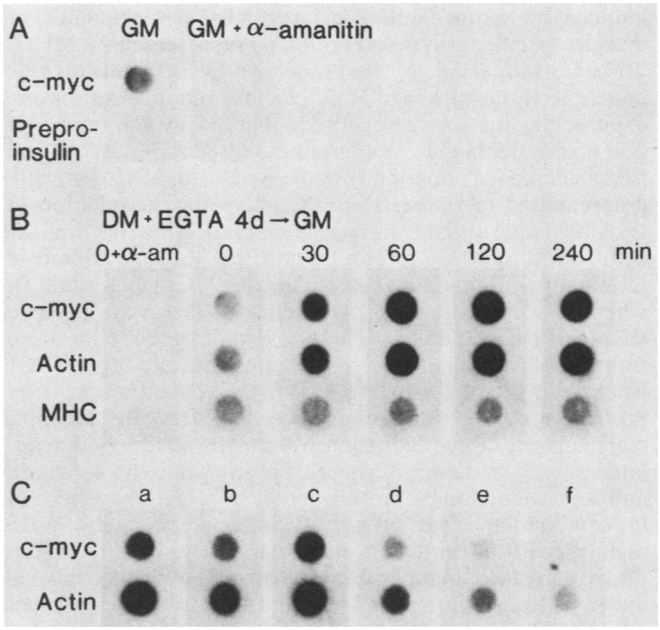

FIG. 7. Nuclear runoff transcription assay of c-*myc* and muscle-specific genes. (A) Nuclei were isolated from L_6E_9-B cells maintained for 3 days in growth medium. Nuclear runoff transcription reactions were conducted in the presence (GM + α-amanitin) or absence (GM) of 1μg of α-amanitin per ml. (B) Nuclei were isolated from L_6E_9-B cells cultured for 4 days in differentiation medium containing 1.75 mM EGTA (0 min), transferred to growth medium, and incubated for 30, 60, 120, and 240 min. Runoff transcription reactions were performed in the presence (0 + α-am) or absence of 1 μg of α-amanitin per ml. (C) Nuclei were isolated from L_6E_9-B cells cultured for 3 days in growth medium (a); cultured for 3 days in growth medium, transferred to fresh growth medium containing 10 μg of cycloheximide per ml, and incubated for 90 min (b); cultured for 4 days in differentiation medium containing 1.75 mM EGTA, switched to growth medium, and incubated for 90 min (c); cultured for 7 days in differentiation medium (d); transferred to growth medium containing 10 μg of cycloheximide per ml (e) or 10 μg/ml anisomycin (f); and incubated for 90 min. Equivalent counts (4×10^5 cpm) of ^{32}P-labeled nuclear runoff transcripts were hybridized with c-*myc* and MHC genomic fragments as well as actin and preproinsulin II cDNAs dot spotted on nitrocellulose filter strips.

induced by serum factors and regulated independently of muscle-specific gene transcription as represented by MHC.

c-*myc* induction by protein synthesis inhibitors is posttranscriptionally regulated. Nuclear runoff transcription experiments further demonstrated that the level of transcription was rather high in proliferating cells (Fig. 7C, lane a) and that it decreased remarkably but was detectable in terminally differentiated myotubes (Fig. 7C, lane d). The addition of cycloheximide or anisomycin decreased c-*myc* as well as actin transcription in proliferating (Fig. 7C, lane b) and in terminally differentiated cell nuclei (Fig. 7C, lanes e and f), whereas it induced or superinduced c-*myc* cytoplasmic mRNA (Fig. 1, 3, and 5). These results, together with those from RNA blotting, indicated that induction of c-*myc* mRNA by growth factors and its induction or superinduction by protein synthesis inhibitors were produced by different mechanisms. c-*myc* induction by growth factors was regulated mainly at the transcriptional level, while the induction and superinduction by protein synthesis inhibitors alone and in combination with growth factors, respectively, were mainly posttranscriptional phenomena. The fact that these drugs increased cytoplasmic c-*myc* mRNA levels but reduced its transcription can be acounted for only by a marked increase in c-*myc* mRNA stability and strongly suggest the inhibition of a short-lived protein that selectively destabilizes this mRNA.

DISCUSSION

There is no relationship between the pattern of c-*myc* gene expression and myogenic cell differentiation. Cell proliferation and induction of the differentiated phenotype appear to be mutually exclusive phenomena in many, if not all, terminally differentiating cell systems (25), including myogenic cells (47). Data from an increasing number of cell types indicate that c-*myc* mRNA is induced early in quiescent cells stimulated to proliferate in response to growth factors (23, 29, 43), whereas its cytoplasmic level decreases markedly in terminally differentiated cells (6, 11, 14, 22, 31, 54, 62). On one hand, these observations have been taken to indicate that a high level of c-*myc* gene expression is necessary for the initiation of DNA synthesis and cell proliferation (29). On the other hand, it has been assumed that its down-regulation is involved in the generation of the quiescent state as well as the induction of differentiation state-specific genes and irreversible withdrawal from the cell cycle (14, 31). The inducibility of biochemical and terminal differentiation in myogenic cells by manipulating Ca^{2+} concentration has made it possible to test these assumptions. The results presented here demonstrate that there were no significant differences between the patterns of c-*myc* expression of biochemically differentiated cells that remain able to reenter the cell cycle and those of terminally differentiated cells that irreversibly withdraw from the cell cycle. In both types of differentiated cells, c-*myc* mRNA exhibited a biphasic induction, remained inducible by serum factors, and was superinduced by the addition of protein synthesis inhibitors. In addition, the patterns of c-*myc* mRNA induction and superinduction in the two types of differentiated cells were not qualitatively different from those in undifferentiated cells, except that the degree of its inducibility by serum in terminally differentiated cells was moderately decreased. Irreversible down-regulation of c-*myc* gene expression did not occur in L_6E_9-B cells and, therefore, cannot be a requirement for their terminal differentiation. Moreover, c-*myc* mRNA deinduction, even for a prolonged period of time, was not sufficient to trigger terminal differentiation, as documented by the biochemically differentiated cells described here and L_6E_9-B cells grown in F12 medium (see reference 52). Under these conditions, cells remained in G_0 phase with a very low level of c-*myc* mRNA and without any evidence of commitment to terminal differentiation (data not shown). In addition, c-*myc* induction in terminally differentiated cells by serum stimulation was not sufficient to trigger DNA synthesis. This result is in agreement with the observation that c-*myc* induction is not sufficient for the mitogenic action of PDGF (8).

Little is known about the mechanisms responsible for the induction of the terminally differentiated myogenic phenotype and its maintenance even in the presence of high concentrations of growth factors. A measured decrease in EGF receptors in the mouse myoblast cell line MM14 during differentiation has led to postulation that the loss of growth factor receptors might be responsible for the inability of the differentiated muscle cells to reenter the cell cycle in response to serum stimulation (33, 34). The present data argue against this interpretation, since it is clear that biochemically and terminally differentiated cells responded to serum growth factors by inducing c-*myc* mRNA, thus suggesting the presence of functional receptors. These terminally differentiated cells, however, were not able to initiate DNA synthesis even in the presence of high concentrations of serum that would presumably provide all known competence and progression factors for cell replication (35, 53, 59), yet mononucleated but biochemically differentiated cells expressing muscle-specific genes in the presence of EGTA were able to reinitiate DNA synthesis by the serum stimulation.

It is possible to argue that a transient decrease in the c-*myc* mRNA level at a particular stage of the differentiation pathway, as has been demonstrated in the present study for myogenic cells and previously for the HL-60 promyelocytic leukemia (62), F9 teratocarcinoma (14), and murine erythroleukemia cells (31), plays a fundamental role in triggering the events leading to biochemical or terminal differentiation. This remains a possibility not excluded by the experiments presented here. However, it has not been previously tested whether there is a causal association between these two phenomena. At least in L_6E_9-B cells under the conditions tested in the present study, changes in c-*myc* expression appear to reflect changes in growth factor levels in the medium rather than the growth rate or differentiated state of the cells. Although it remains to be determined whether cytoplasmic mRNA levels under normal conditions reflect c-*myc* protein synthesis, from the experiments described here there is no evidence for a direct role of c-*myc*, either positive or negative, in the induction or maintenance of the irreversible withdrawal of myoblast from the cell cycle, which is essential for terminal differentiation (47). Although inhibition of myogenesis in avian cells transformed by myelocytomatosis virus MC29 that carries v-*myc* has been reported (17), the results presented here clearly demonstrated that continuous c-*myc* gene transcription and high cytoplasmic levels of its mRNA did not have any detectable effect on the transcription of muscle-specific genes and their mRNA accumulation. It remains to be determined whether the different effects of v-*myc* and c-*myc* on myogenesis and muscle-specific gene expression are due to qualitative differences between the two oncogenes or are a reflection of their respective levels of expression.

c-*myc* mRNA induction by growth factors and protein synthesis inhibitors is regulated at the transcriptional and posttranscriptional level, respectively. Leder et al. (32) have

proposed that c-*myc* gene is regulated by a *trans*-acting negative control element that acts on two discrete transcriptional promoters. This hypothesis is favored by the fact that, in some Burkitt lymphomas or mouse plasmacytomas, the promoters of translocated c-*myc* genes are damaged or lost, and the transcription of such c-*myc* genes is deregulated. Moreover, c-*myc* mRNA is induced by a protein synthesis inhibitor, cycloheximide, and superinduced by cycloheximide in combination with growth factors or liver regeneration (29, 37, 43), implying that the c-*myc* mRNA level is regulated by a labile protein. This labile protein has been postulated to act as a *trans*-acting repressor, but the possibility can not be ruled out that cycloheximide stabilizes c-*myc* mRNA by inhibiting the synthesis of a labile protein that destabilizes c-*myc* mRNA. During the preparation of this manuscript, several reports have appeared indicating that posttranscriptional control is responsible for the regulation of c-*myc* mRNA levels in interferon-treated Daudi cells (30), in murine plasmacytomas (51), in response to growth factors in G_0-arrested fibroblasts (4), and in differentiating F9 tetatocarcinoma cells (14).

The results presented here indicated that in L_6E_9-B cells both transcriptional and posttranscriptional mechanisms were involved in regulating c-*myc* mRNA levels. Contrary to the results obtained from G_0-arrested fibroblasts (4), transcriptional control is sufficient to account for the level of cytoplasmic c-*myc* mRNA induction in L_6E_9-B cells in response to growth factor stimulation. In quiescent biochemically differentiated cells, there was a good correlation between the 5- to 10-fold increase in nascent transcripts in the nuclei isolated from serum-stimulated cells and the 5- to 10-fold induction of cytoplasmic c-*myc* mRNA. These results indicate that, although the half-life of the mRNA is very short (10, 39, 51), posttranscriptional mechanisms need not be invoked to explain this induction.

In addition to this transcriptional regulation by serum factors, the induction of c-*myc* mRNA by protein synthesis inhibitors alone and its superinduction by the drugs combined with serum indicated that a short-lived protein factor(s) acted as a negative regulator for c-*myc* expression in proliferating, quiescent, and terminally differentiated cells. Since c-*myc* transcription declined in the nuclei of cells treated with cycloheximide or anisomycin, the induction and superinduction of its mRNA can be explained only by a remarkable increase in its stability. This effect is most likely due to the selective stabilization of c-*myc* mRNA occurring when the synthesis of a labile negative regulatory protein is inhibited. This protein appears to be constitutively expressed in L_6E_9-B cells since, in all cases tested, protein synthesis inhibitors induced c-*myc* mRNA but not muscle-specific protein mRNAs. A similar mechanism of posttranscriptional regulation has been described for HeLa cells, breast carcinoma cells, promyelocytic leukemia cells (10), and chinese hamster lung fibroblasts (4). Recently, Piechaczyk et al. (51) have reported that transcription rates of truncated c-*myc* gene in mouse plasmacytomas are comparable to those of the intact c-*myc* gene but that mRNA from truncated c-*myc* gene has a significantly longer half-life. This fact points toward the sequences encoded by the first exon of the gene as the target for the negative regulatory factor(s) (32). This hypothesis, however, does not account for the fact that cycloheximide has no effect on c-*myc* mRNA stabilization in Daudi cells and human embryo fibroblasts (10), which presumably have an intact c-*myc* gene.

These results, together with the data presented in this paper, indicate that c-*myc* can be regulated at both transcriptional and posttranscriptional levels. The relative predominance of each of these two mechanisms appears to vary among cell types, the nature of the inducers, and the physiological states of the cells. The heterogeneity of expression and regulation of this gene among cell types makes it difficult to draw general conclusions from the individual cell types. Elucidation of the physiological significance of these different modes of regulation most probably will require a better understanding of the function of this gene product in cellular processes.

ACKNOWLEDGMENTS

We thank M. Fennell for expert secretarial assistance.

This work was supported by grants from the National Institute of Health and the Muscular Dystrophy Association of America.

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