Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 5323–5327, August 1985
Biochemistry

c-*myc* mRNA levels in the cell cycle change in mouse erythroleukemia cells following inducer treatment

(oncogenes/elutriation/terminal differentiation/proliferation)

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Communicated by Harry Eagle, May 6, 1985

ABSTRACT **Several lines of evidence suggest that the c-*myc* protooncogene is involved in some aspect of cell division in mammalian cells. We have been investigating changes in the expression of c-*myc* mRNA in mouse erythroleukemia cells during chemically induced terminal erythroid differentiation. *In vitro* induction of erythroleukemia cell differentiation results in a switch from cells with unlimited proliferative capacity to cells that undergo a small number of terminal cell divisions. The level of c-*myc* mRNA changes rapidly following treatment with inducing agents. After a very rapid decline the mRNA is restored to pretreatment levels and then declines again. We have now measured the level of c-*myc* mRNA with respect to position in the cell cycle. Prior to inducer treatment the level of c-*myc* mRNA is relatively constant throughout the cell cycle. However, when the mRNA is restored following treatment with hypoxanthine or hexamethylenebisacetamide, it is found primarily in cells in the G_1 phase. Thus, treatment with inducers of differentiation leads to a change in the cell cycle regulation of c-*myc* mRNA. This change may be involved in the altered proliferative capacity of the cells that occurs during terminal differentiation.**

The c-*myc* protooncogene is the cellular homologue of v-*myc*, the transforming gene from the MC29 retrovirus (1, 2). Unlike the *ras* family of oncogenes in which mutations in the coding sequence result in expression of an abnormal protein that is involved in malignant transformation (3–5), in many cases the c-*myc* gene product appears to be normal in tumors in which c-*myc* has been suggested to be responsible for transformation (6). Instead, the transformed state of these tumor cells appears to be associated with abnormal regulation of c-*myc* gene expression (7–10). For example, in murine and human B-cell tumors abnormal expression of c-*myc* most frequently has been observed to result from translocation of c-*myc* coding sequences within immunoglobulin loci (11–14).

The physiological role of c-*myc* is poorly understood. c-*myc* mRNA is readily detected in most normal, dividing cells (15). The mRNA is rapidly induced in normal resting fibroblasts and lymphocytes after treatment with mitogens that induce cell division (16, 17). Maximal induction is achieved prior to the onset of DNA synthesis, suggesting that c-*myc* may be involved in an early step in the decision to transit a new cell cycle. The demonstration that transformation of secondary rat embryo fibroblasts by the EJ H-*ras*-1 oncogene requires coexpression of c-*myc*, whereas it is not needed for transformation of established cell lines, further suggested that c-*myc* may be involved in "immortalization" of cell lines (18). The finding that a decrease in c-*myc* expression occurs during terminal differentiation of several types of tumor cells is also consistent with the idea that c-*myc* is involved in regulating cell division. c-*myc* mRNA levels were found to decline during differentiation of the HL-60 leukemic line (19, 20) and in F9 teratocarcinoma cells (21). c-*myc* mRNA levels have also been reported to decline during receptor-mediated growth inhibition in a murine B-cell lymphoma line (22).

We recently reported that the level of c-*myc* mRNA undergoes dramatic changes during chemically induced differentiation of mouse erythroleukemia (MEL) cells (23). These cells are transformed erythroid lines that are blocked at an early stage of differentiation. Upon treatment with a wide variety of chemicals, the cells are induced to reinitiate a differentiation program that culminates in extensive hemoglobin synthesis (reviewed in ref. 24). During this process the cells lose their tumorigenic potential (25) and ultimately cease proliferating *in vitro*. Clonal assays indicate that cells that are irreversibly committed to terminal differentiation can first be detected 12–24 hr after addition of an inducer and that most cells are so committed by 24–48 hr (26). We found that c-*myc* mRNA levels decreased by a factor of about 10–15 within 2 hr after exposure to several inducers (23). Curiously, however, c-*myc* mRNA was later restored to pretreatment levels or higher between 4 and 18 hr and then declined once again after 24 hr as cells underwent terminal differentiation. The precise kinetics of c-*myc* mRNA restoration were dependent upon the particular chemical inducer used, but it has been observed with both dimethyl sulfoxide and hexamethylenebisacetamide (HMBA) as well as with the chemically unrelated inducer hypoxanthine.

Although the levels of c-*myc* mRNA achieved during its restoration are equal to or greater than the levels observed in uninduced cells, we considered the possibility that the cell cycle distribution of c-*myc* mRNA may be different from uninduced MEL cells. We therefore have measured the level of c-*myc* mRNA in the cell cycle both before and during MEL cell differentiation. We report here experiments indicating that c-*myc* mRNA levels vary about 2-fold throughout the cell cycle in proliferating, uninduced MEL cells, whereas its restoration in inducer-treated MEL cells is more highly restricted to cells in the G_1 phase.

METHODS

Cell Culture and Synchronization. MEL cells (clone 745) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 100 units of penicillin/streptomycin per ml. To induce differentiation cells were grown to a density of $5–10 \times 10^5$ cells per ml and diluted to $0.5–1.0 \times 10^5$ cells per ml by adding fresh medium containing either hypoxanthine or HMBA at a final concentration of 5 mM. Cells in the various stages of the cell cycle were prepared by centrifugal elutriation as described with the exception that a JE-10X rotor was used (27). Cells ($1–3 \times 10^9$) were harvested for each elutriation. The average

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Abbreviations: MEL, mouse erythroleukemia; HMBA, hexamethylenebisacetamide; FMF, flow microfluorimetry.

DNA content of cells in each fraction was determined by flow microfluorometric analysis of propidium iodide-stained nuclei with a FACS II unit. The DNA content is expressed as the modal C value, where C is the haploid DNA content of cells in G_1 phase. The percentage of cells undergoing DNA synthesis was determined by autoradiography after pulse-labeling aliquots of the elutriated cell fractions with 2 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine per ml for 20 min at 37°C.

Preparation and Analysis of RNA. Total cellular RNA was prepared by hot phenol extraction as described (28). Twenty micrograms of total cellular RNA was fractionated on 0.9% agarose gels containing 3% formaldehyde. RNA was transferred to nitrocellulose filters according to the method of Thomas (29). The prehybridization and hybridization conditions used have been described (23). Hybridization probes were labeled by nick-translation with [^{32}P]TTP and [^{32}P]dCTP to a specific activity of 1–3 $\times$ 10^8 dpm/μg. Following autoradiography, the hybridizing bands were scanned with a Joyce–Loebl densitometer and the areas under the curves were measured with a Numonics Corporation integrator. Autoradiograms within the linear range of intensity were subjected to densitometric analysis.

RESULTS

c-*myc* mRNA Levels Throughout the Cell Cycle in Uninduced MEL Cells. To investigate the cell cycle distribution of c-*myc* mRNA in uninduced MEL cells, an exponentially growing culture of cells was separated into cell cycle-specific fractions by centrifugal elutriation. This technique separates cells at various positions in the cell cycle on the basis of differences in size, which is correlated with DNA content. The technique has the advantage of yielding relatively pure cell cycle-specific fractions without requiring the use of biochemical agents to synchronize the cells. The properties of the fractions obtained by elutriation were examined by determining the relative DNA content by flow microfluorimetry (FMF) and by measuring the proportion of cells undergoing DNA synthesis by pulse-labeling with [^{3}H]thymidine. The elutriated MEL cell fractions exhibited incremental increases in DNA content from 2C (G_1 fraction) to 4C (G_2 fraction). There was usually <10–20% contamination of the G_1 fraction with S-phase cells (data not shown). Total cellular RNA was extracted from nine elutriated fractions whose modal cellular DNA contents are indicated on the abscissa in Fig. 1. Equal amounts of these RNA samples were separated on an agarose/formaldehyde gel and hybridized after transfer to nitrocellulose filters. Fig. 1*A* (upper panel) is a photograph of the ethidium bromide-stained gel showing that relatively constant amounts of RNA were electrophoresed in each lane. The 2.3-kilobase c-*myc* mRNA was detected by hybridizing with a mouse cDNA clone, pM-c-myc54 (30). The relative levels of c-*myc* mRNA in each fraction were determined by densitometric scanning of the autoradiographic bands [Fig. 1 *A* (middle panel) and *B*]. In this experiment the highest amount of c-*myc* mRNA was seen in cells in G_1 phase. The level was ≈2-fold higher than the lowest levels found in middle to late S phase (DNA content, 2.5–3.9C). We performed three separate elutriation experiments with uninduced MEL cells and prepared duplicate RNA transfer filters in each experiment. In these experiments the levels of c-*myc* mRNA in S-phase cells ranged between 50% and 80% of that found in G_1 phase.

To compare the levels of c-*myc* mRNA with another mRNA species whose cell cycle regulation is well established, the filter shown in the middle panel of Fig. 1*A* was rehybridized with a mouse H3 histone probe. As visualized in the lower panel of Fig. 1*A* and demonstrated by the densitometric analysis (Fig. 1*B*), the level of H3 histone mRNA was about 8-fold higher in S-phase cells compared

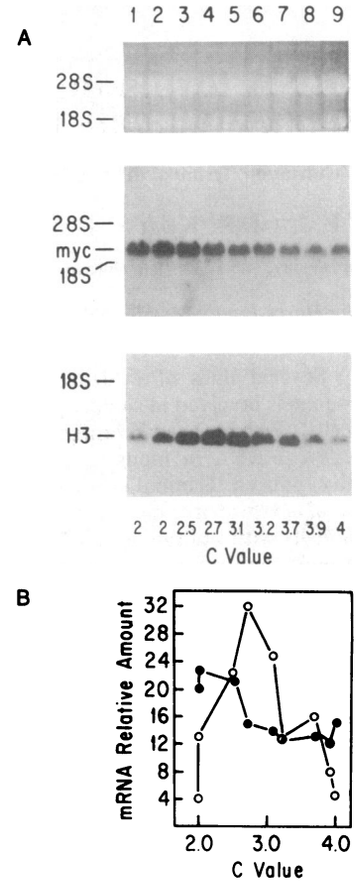

FIG. 1. Relative levels of c-*myc* and histone H3 mRNAs in elutriated, uninduced MEL cells. (*A*) Uninduced MEL cells were separated into nine cell cycle fractions by centrifugal elutriation. The average modal DNA content of cells, expressed as a C value, where C is the haploid DNA content of cells in G_1 phase, was determined by FMF. The C value in each fraction is shown below the lower panel. Twenty micrograms of RNA from each fraction was fractionated on a formaldehyde/agarose gel. Upper panel: the 18S and 28S ribosomal RNA bands were visualized by staining with ethidium bromide. Middle panel: RNA blot hybridization with a mouse c-*myc* probe. The RNA from the gel shown in the upper panel was transferred to nitrocellulose filters. RNA blot hybridization was performed by using ^{32}P-labeled pM-c-myc54 as a probe. The autoradiographic exposure time was 24 hr. Lower panel: RNA blot hybridization with a mouse histone H3 probe. The filter shown in the middle panel was rehybridized with ^{32}P-labeled pRAH3-2 (29). Autoradiographic exposure time was 5 hr. (*B*) The hybridizing bands obtained in *A* were scanned by a Joyce–Loebl densitometer. The areas of the densitometric tracings were used to calculate relative mRNA levels. ●, c-*myc* mRNA; ○, histone H3 mRNA. The levels for histone and c-*myc* mRNAs cannot be directly compared since the data points for the two curves were not normalized to account for differences in probe specific activities and autoradiographic conditions.

with cells in G_1 or G_2 phase. This result is consistent with our published data (31). Thus, in contrast to histone H3 mRNA levels, which vary periodically in the cell cycle, the concentration of c-*myc* mRNA in uninduced MEL cells varies only 2-fold throughout the cell cycle.

The Distribution of c-*myc* mRNA in the Cell Cycle Changes Following Treatment with an Inducer. Upon treatment with several different inducers of MEL cell differentiation, c-*myc* mRNA levels abruptly decline. However, after 4–18 hr of treatment, depending on the particular inducer used, c-*myc* mRNA is restored to pretreatment levels or greater (23). When hypoxanthine was used to induce differentiation, c-*myc* mRNA was restored rapidly after 4 hr and was

maintained at this level until about 10 hr, at which time it was observed to decline (Fig. 2*A*). To determine whether c-*myc* mRNA was restored in all cells regardless of cell cycle position or in cells traversing a particular phase of the cycle, MEL cells were treated with hypoxanthine for 8 hr and then elutriated to obtain cell cycle-specific fractions. Constant amounts of total cellular RNA were electrophoresed in agarose/formaldehyde gels. A photograph of one such gel stained with ethidium bromide is shown in the upper panel of Fig. 2*B*. The levels of c-*myc* and H3 histone mRNA were determined by RNA transfer hybridization as described above. In contrast to uninduced MEL cells in which there was only a 2-fold difference in c-*myc* mRNA levels throughout the cell cycle, cell cycle-specific fractions obtained from MEL cells treated with hypoxanthine exhibited a 10-fold higher level of c-*myc* mRNA in G_1 phase compared to the lowest level found in cells near the middle of S phase [Fig. 2 *B* (middle panel) and *C*]. We also observed a similar difference between the maximal value in G_1 phase and that in middle S phase in a separate elutriation of hypoxanthine-treated MEL cells. In both experiments, duplicate or triplicate RNA transfers were analyzed and similar results were obtained. Therefore, when c-*myc* mRNA is restored following hypoxanthine treatment, the mRNA is found in a more restricted portion of the cell cycle as compared with uninduced MEL cells.

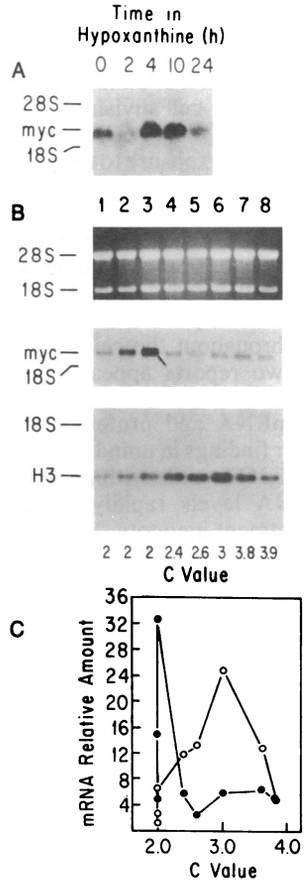

FIG. 2. Relative levels of c-*myc* and histone H3 mRNAs in hypoxanthine-treated MEL cells. (*A*) mRNA levels in unfractionated cells. MEL cells were grown in the presence of 5 mM hypoxanthine for 24 hr. At the indicated times RNA was prepared from aliquots of cells and the level of c-*myc* mRNA was determined by RNA blot hybridization. Autoradiographic exposure time was 48 hr. (*B*) MEL cells were grown in the presence of 5 mM hypoxanthine for 8 hr, at which point c-*myc* mRNA had reaccumulated (see *A*). The cells were separated into eight cell cycle-specific fractions by elutriation (DNA content below lower panel) and the levels of c-*myc* and histone H3 mRNAs were determined as described in the legend to Fig. 1. Upper panel: ethidium bromide stain showing 18S and 28S ribosomal RNA bands. Middle panel: the RNA in the upper panel was transferred to nitrocellulose and hybridized with pM-c-myc54. Autoradiographic exposure time was 16 hr. Lower panel: histone H3 mRNA; autoradiographic exposure time was 16 hr. (*C*) The hybridizing bands in *B* were subjected to densitometric analysis and the areas under the tracings were plotted as a function of DNA content. ●, c-*myc* mRNA; ○, histone H3 mRNA.

In contrast to the change in the distribution of c-*myc* mRNA in the cell cycle that occurred following hypoxanthine treatment, the predominance of histone H3 mRNA in early to middle S phase seen in uninduced cells was maintained in hypoxanthine-treated cells [Fig. 2 *B* (lower panel) and *C*].

The level of c-*myc* mRNA in the G_1 fraction shown in lane 3, Fig. 2*B*, is 2- to 6-fold higher than the level found in earlier G_1 fractions (lanes 1 and 2). Although the modal DNA contents of these three fractions is 2C, they contain populations of G_1-phase cells that differ in size. A study comparing cell size with DNA content has demonstrated that, beginning after mitosis, there is an increase in cell diameter of about 8% that precedes the onset of DNA synthesis (unpublished data). Thus, the RNA samples in lanes 1–3 were obtained from cells in the initial and late stages of G_1 phase. We observed a consistently higher level of c-*myc* mRNA in late G_1-phase cells compared with that found in early G_1 phase following treatment with hypoxanthine. The results suggest that after inducer treatment c-*myc* mRNA accumulates during the course of G_1 phase.

To determine whether the restricted accumulation of c-*myc* mRNA in G_1 phase also occurs with other inducers, we determined the relative levels of c-*myc* mRNA following treatment with HMBA, a potent inducer of MEL cell differentiation. The change in the level of c-*myc* mRNA following HMBA treatment differed from that observed after hypoxanthine treatment in that the reappearance of c-*myc* mRNA occurred later, beginning between 8 and 12 hr, and reached a maximal level at about 16 hr. To determine whether c-*myc* mRNA was restored primarily in G_1 phase following HMBA treatment we used a different experimental approach from the experiment shown in Fig. 2. Purified populations of MEL cells in G_1 phase, middle S phase, and late S/G_2 phase were prepared by centrifugal elutriation of an uninduced culture of MEL cells. Each cell population was incubated at 37°C in the presence of 5 mM HMBA and total cellular RNA was extracted every 4 hr during 24 hr of HMBA treatment. Parallel samples also were analyzed for DNA content by FMF. The FMF profiles obtained from one experiment are shown in Fig. 3. Previous studies have shown that following treatment with several different inducers MEL cells exhibit a prolonged G_1 phase period (32, 33). Hypoxanthine treatment does not result in as pronounced a prolongation of G_1 phase as occurs with other inducers (33). The prolonged G_1-phase period is reflected in the FMF profiles shown in Fig. 3. In the fraction of cells that was in G_1 phase when HMBA was added (left panel, Fig. 3), some cells did not move from G_1 phase following HMBA treatment. However, most cells traversed the cell cycle and exhibited the prolonged G_1 phase beginning at about 16 hr as described. Cells originally in middle S phase when HMBA was added (middle panel, Fig. 3) moved through S and G_2 phases and accumulated in G_1 phase at about 8 hr. The prolonged G_1 phase lasted until about 20 hr, at which time a fraction of the cells underwent a new round of DNA synthesis. Finally, in the fraction of cells that was in late S phase and G_2 phase when HMBA was added (right panel, Fig. 3), most cells had reached G_1 phase by 8 hr. About 50% of these cells appeared to arrest in G_1 phase, whereas the remaining cells traversed the cell cycle and reached the subsequent G_1 phase at about 16 hr. Thereafter

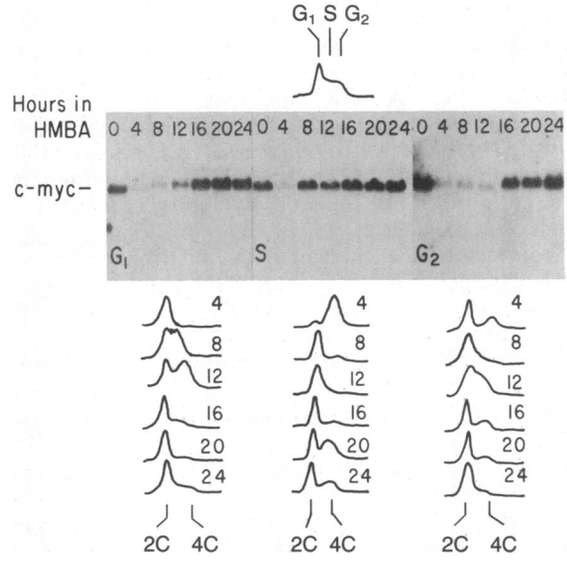

FIG. 3. Kinetics of reappearance of c-*myc* mRNA following HMBA induction of synchronized cells. Uninduced MEL cells were fractionated into cell cycle-specific fractions by centrifugal elutriation. Fractions representing cells in G_1 phase (DNA content, 2.0C), middle S phase (DNA content, 3.2C), and a combined late S-phase + G_2-phase fraction (DNA content, 3.9–4.0C) were grown in the presence of 5 mM HMBA for 24 hr. Aliquots of cells were removed at the indicated times and the FMF profiles shown below each autoradiogram were obtained. The level of c-*myc* mRNA was determined as described in the legend to Fig. 1. Autoradiographic exposure time was 48 hr. The filter whose autoradiogram is seen in the right panel (late S + G_2 phases) was produced in a separate hybridization experiment from that used to prepare the left and middle panels.

this fraction of cells arrested in G_1 phase for the duration of the 24-hr experiment.

As seen in the autoradiogram in Fig. 3, each of the cell cycle fractions exhibited a decline in c-*myc* mRNA by a factor of about 10 at 4 hr after the addition of HMBA. This decline was similar to that observed in unsynchronized populations of MEL cells treated with inducers and demonstrated that the decline of c-*myc* mRNA occurred in all cells regardless of their position in the cell cycle. However, the timing of the reappearance of c-*myc* mRNA was different in each of the cell cycle fractions.

Cells that were in G_1 phase when HMBA was added started to accumulate c-*myc* mRNA 12 hr later and reached pretreatment levels at 16 hr, when all of the cells had entered G_1 phase. Cells originally in late S/G_2 phase when HMBA was added also reaccumulated c-*myc* mRNA after 16 hr, at the time when a large percentage of cells were in G_1 phase. Note that these cells did not contain high levels of c-*myc* mRNA at 8 hr, when most of the cells were in the first G_1-phase period. Reappearance of c-*myc* mRNA in this fraction appeared to correlate with the time when cycling cells entered a second G_1 phase. However, when HMBA was added to cells in S phase, the reappearance of c-*myc* mRNA occurred earlier at about 8 hr. This time corresponded to the point at which most cells had entered G_1 phase.

These data indicate that the reappearance of c-*myc* mRNA does not simply occur after a fixed time has elapsed in the presence of inducer. Rather, the mRNA accumulates as cycling cells enter a new G_1 phase after the addition of inducer. In cultures originally in G_1 phase when HMBA was added, there was a fraction of cells that remained in G_1 phase without undergoing a round of DNA synthesis. These cells did not appear to reaccumulate c-*myc* mRNA since the mRNA was no more than 10% of the maximal levels at this time. It is interesting to consider why these cells that were prematurely arrested in G_1 phase did not contain high levels of c-*myc* mRNA. Perhaps the cells must undergo a round of DNA synthesis before the level of c-*myc* mRNA can be restored. The fact that G_2-phase cells treated with HMBA also failed to reaccumulate c-*myc* mRNA during the first G_1 phase and, rather, appeared to require a complete cell cycle for restoration is consistent with this suggestion.

By using a modified experimental approach and a different inducer of MEL cell differentiation, the data with HMBA (Fig. 3) confirm those obtained with hypoxanthine. Both studies show that after the addition of inducing agents, c-*myc* mRNA reaccumulates primarily in cells in the G_1 phase.

DISCUSSION

The molecular mechanisms involved in the irreversible commitment of MEL cells to differentiate are not well understood. Commitment occurs when a cell is able to complete the differentiation program in the absence of the inducer. Differentiation of MEL cells and other types of tumor lines is associated with a switch from unlimited proliferative potential to a set of terminal cell divisions. Previous work has shown that induction of MEL cell commitment requires a minimal latent period of exposure to the inducer and that after commitment occurs cells are capable of four or five additional cell divisions (26). The majority of committed MEL cells appears after 24 hr of exposure to inducers. The results reported here show that prior to this time, a change occurs in the cell cycle distribution of c-*myc* mRNA. In proliferating, uninduced MEL cells c-*myc* mRNA levels were found not to vary by >2-fold throughout the cell cycle. After this work was completed, two reports appeared demonstrating that other types of proliferating cells contain relatively constant levels of c-*myc* mRNA and protein in the cell cycle, in agreement with our findings in uninduced MEL cells (34, 35). Upon treatment of MEL cells with an inducer of differentiation c-*myc* mRNA levels rapidly decline and then are restored to pretreatment amounts. We showed in two ways that the restoration of c-*myc* mRNA occurs primarily in cells in the G_1 phase of the cell cycle. Eight hours after the addition of the inducing agent hypoxanthine, we observed a large difference (10-fold) between the level of c-*myc* mRNA in cells in G_1 phase and the level in cells in middle S phase. By using synchronized populations of cells we also showed that HMBA-treated cells reaccumulate c-*myc* mRNA as they enter G_1 phase. Thus, treatment of MEL cells with inducers of differentiation appears to lead to a restricted accumulation in the level of c-*myc* mRNA during G_1 phase.

The change in the cell cycle distribution of c-*myc* mRNA may reflect differences in the proliferative potential of uninduced and committed MEL cells. Unlimited proliferative potential may be favored by cells that maintain relatively constant levels of c-*myc* mRNA throughout the cell cycle, whereas entry into a terminally differentiated program may occur in cells in which c-*myc* mRNA is restricted in G_1 phase. Our results suggest that an important aspect of c-*myc* expression may be its timing in the cell cycle or in the differentiation program rather than simply its level of expression.

Certain earlier studies suggested that translocation of the c-*myc* gene to immunoglobulin loci in murine plasmacytomas results in greatly increased expression of c-*myc* relative to that in nontransformed cells (13, 36). However, when the comparison is made with nontransformed cells that are proliferating it is apparent that the tumor cells do not have substantially higher levels of c-*myc* mRNA. For example, Keath *et al.* (7) showed that the level of c-*myc* mRNA in several mouse plasmacytomas was similar to that in mitogen-stimulated B lymphocytes. Likewise, Campisi *et al.* (21)

found that c-*myc* mRNA levels in proliferating nontransformed A31 fibroblasts were similar to those in chemically transformed derivatives. However, these investigators did observe a difference in the cell cycle regulation of c-*myc* expression in the two types of fibroblasts. When nontransformed fibroblasts were made quiescent by serum deprivation they exhibited very reduced levels of c-*myc* mRNA. Transformed fibroblasts treated in the same way maintained c-*myc* mRNA levels comparable to that of the proliferating cells. In this paper we describe another example in which there is a change in the cell cycle regulation of c-*myc* mRNA. In this case the change is associated with a transition from continuously dividing cells to cells that are destined to undergo a limited number of cell divisions.

We thank Dr. Ken Marcu for supplying pM-c-myc54, Ann Gorgoglione for typing the manuscript, and Valerie Didamo for excellent technical assistance. This work was supported by National Institutes of Health Grants PHS CA 16368 (A.I.S.), GM 22332 (C.L.S.), and CA13330 and by ACS Grant CD239 (C.L.S.). K.S.H. is a recipient of a National Institutes of Health Fellowship.

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