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c-*myc* Regulation during Retinoic Acid-Induced Differentiation of F9 Cells Is Posttranscriptional and Associated with Growth Arrest

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We have shown that c-*myc* mRNA levels decrease more than 20-fold when F9 teratocarcinoma stem cells are induced to arrest growth and terminally differentiate to parietal endoderm after exposure to retinoic acid and cyclic AMP (Campisi et al., Cell 36:241–247, 1984). Here, we demonstrate that although growth arrest and full expression of the differentiated phenotype required about 3 days, c-*myc* mRNA declined abruptly between 8 and 16 h after the addition of retinoic acid and cyclic AMP. The decline was independent of cyclic AMP. We found little or no change in the level of c-*myc* transcription during differentiation, although two other genes showed marked transcriptional regulation. Thus, decreased c-*myc* mRNA is a consequence of very early posttranscriptional regulation directed by retinoic acid. Differentiation was not fundamental to this regulation. We have shown that sodium butyrate blocks expression of the differentiated phenotype if added within 8 h of retinoic acid and cyclic AMP (Levine et al., Dev. Biol. 105:443–450, 1984). However, butyrate did not inhibit the decrease in c-*myc* mRNA. Furthermore, F9 cells partially arrested growth without differentiating when grown in isoleucine-deficient medium. Under these conditions, c-*myc* mRNA levels also declined. Our results suggest that induction of differentiation-specific genes may be under retinoic acid-mediated control dissimilar from that responsible for the decay of c-*myc* mRNA. In addition, they raise the possibility that growth arrest may be initiated by reduced c-*myc* expression.

Differentiation is frequently associated with an altered pattern of growth (10, 21). An extreme example of the coupling of growth and differentiation is terminal differentiation, whereby cells irreversibly lose proliferative capacity in addition to expressing new structural genes. How changes in growth potential are related to expression of the differentiated phenotype is not known. Cancer cells, which have lost growth control, often display a more embryonic phenotype (35), suggesting that the growth pattern and differentiated characteristics are related. Some transformed cells in culture fail to terminally differentiate in response to appropriate signals (37, 50). However, others can be induced to terminally differentiate in culture (8, 15, 32). In these examples, growth and differentiation appear to have been uncoupled (39).

Proto-oncogenes comprise a class of cellular genes that may regulate growth and differentiation. When mutated or aberrantly expressed, they are thought to play pivotal roles in establishing or maintaining (or both) the tumorigenic phenotype (4). The *myc* proto-oncogene (c-*myc*) is expressed in many cell types, both normal and tumor derived (4, 31, 49). c-*myc* may play a role in regulating cell proliferation (1), although how it functions in this regard is unknown. The mRNA level declines when immortal 3T3 fibroblasts are made quiescent in culture (6); it rises rapidly and manyfold when quiescent 3T3 cells or lymphocytes are induced to proliferate (23) and in the early stages of liver regeneration (17, 33). Thus, during proliferative changes, expression is tightly regulated.

Unregulated c-*myc* expression is common in some leukemias and lymphomas; these tumor cells are highly proliferative and are often blocked at an earlier stage than the terminal stage of differentiation (28, 49). In addition, we have shown that c-*myc* mRNA abundance greatly diminishes after murine F9 embryonal carcinoma cells are induced by retinoic acid and cyclic AMP to terminally differentiate into parietal endoderm (6). Terminal differentiation of hematopoietic cells is also accompanied by decreased c-*myc* expression (16, 25, 36, 49). These findings raise the possibility that this proto-oncogene may participate in the regulation of differentiation.

In this study, we examined c-*myc* mRNA levels in F9 cells during manipulations of growth and differentiation. Retinoic acid induced an early, posttranscriptional change in *myc* gene expression, and this correlated with growth arrest rather than differentiation. Thus, mutation or loss of control of this gene could uncouple growth and differentiation.

MATERIALS AND METHODS

Cell culture. F9 cells (3) were obtained and grown on gelatinized plasticware in Dulbecco modified Eagle medium (DME) supplemented with 4 mM glutamine and 10% donor calf serum as previously described (6). Retinoic acid (RA) was prepared weekly as a 5 mM stock solution in ethanol, dibutyryl cyclic AMP was prepared as a 200 mM aqueous solution stored frozen and thawed only once, and theophylline was a 100 mM stock in 0.1 N NaOH. Appropriate dilutions of ethanol or NaOH had no effect on the cells.

Cultures were started from frozen stocks every 10 to 12 weeks and were checked for contaminating mycoplasma by the ratio of incorporated [^{3}H]uridine to [^{3}H]uracil (40).

Growth curves. Cells (3×10^4) were plated onto gelatinized 35-mm culture dishes. After 24 h the medium was replaced with experimental medium, which was again replenished after 3 days. At the indicated times, the cells were

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trypsinized, diluted into Hanks salts plus 0.25% Formalin, and counted with a Coulter electronic particle counter.

RNA isolations. Total cellular RNA was isolated by lysis in guanidine hydrochloride as described by Cox (9) and Strohman et al. (45) and quantitated by absorbance at 260 nm. Quantitation was confirmed by electrophoretically fractionating a sample on formaldehyde-agarose gels, staining with ethidium bromide, and observing the intensities of the rRNA bands.

DNA probes. The M13-mp8-myc clone used for S1 analysis was constructed by inserting into the M13-mp8 vector the *Bam*HI-*Hin*dIII fragment of a murine c-*myc* gene containing exons 2 and 3 (M. Dean, Ph.D. thesis, Boston University Medical School, 1983). The *myc* probes used for transcription (with comparable results) were either a 5.5-kilobase *Bam*HI fragment of a murine c-*myc* kindly provided by P. Leder, Harvard University Medical School (24), or a murine c-*myc* cDNA kindly provided by K. Marcu, State University of New York, Stony Brook (41). The actin probe was a 1-kilobase cDNA rat clone, generously provided by S. Farmer (5), and the collagen IV probe was a cDNA clone from F9 cells, kindly donated by S. Farmer, R. Niles, and B. Smith of Boston University Medical School; this clone was isolated by using the collagen IV cDNA clone described by Marotti et al. (34), and its identity was confirmed by sequencing (Farmer, Niles, and Smith, personal communication). The isolation and characterization of pST6-135 have been previously described (30).

RNA analysis. S1 nuclease quantitation was performed by the method of Berk and Sharp (2) as modified by Favalaro et al. (13). The probe was either the 9-kilobase *Bss*HII fragment or the 0.45-kilobase *Bss*HII-*Sac*I fragment of M13-mp8-myc. The DNA was end labeled for 15 min at 22°C with 5 to 10 U of DNA polymerase I (Klenow fragment) and 100 μCi of [^{32}P]dCTP (800 Ci/mmol) in 6 mM Tris hydrochloride (pH 7.5)–6 mM $MgCl_2$–50 mM NaCl–1 mM β-mercaptoethanol–100 μM GTP. The reaction sample was extracted with phenol, precipitated twice with ethanol, and suspended in hybridization buffer [80% formamide, 400 mM NaCl, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.4), 1 mM EDTA]. The probe (10 μl; 20,000 to 100,000 dpm) was hybridized to 10 to 25 μg of RNA at 64°C for 18 h. Samples were digested for 20 min at 37°C with 300 U of S1 nuclease per ml. Undigested fragments were precipitated with ethanol, suspended in 90% buffered formamide, and electrophoresed on 8 M urea–5% acrylamide gels. The gels were autoradiographed with preflashed film, and autoradiograms were quantitated by densitometry.

RNA blot analysis was performed as described by Dean et al. (12).

Nuclear runoff assays. Nuclei were isolated and analyzed for runoff transcripts hybridizable to cloned DNA by modification of the method of Greenberg and Ziff (18). Briefly, cells were lysed in Nonidet P-40 buffer, and the nuclei were pelleted and frozen in liquid N_2. For the transcription assay, nuclei (10^7) were thawed and incubated in reaction buffer containing 100 μCi of [α-^{32}P]UTP. Reaction kinetics were linear for at least 30 min, and incorporation into *myc*-hybridizable RNA was inhibited by α-amanitin as reported previously (18). After 30 min, labeled RNA was isolated. DNA was immobilized on nitrocellulose filters, which were prehybridized overnight in hybridization buffer containing additional 2× Dennardt solution, 0.05% pyrophosphate, 50 μg of *Escherichia coli* tRNA per ml, and 50 μg of salmon sperm DNA per ml. We found that prehybridization greatly improved the reproducibility of these assays. Hybridization was carried out at 65°C for 40 h. The filters were washed, treated with RNase, dried, and autoradiographed onto preflashed film.

Flow microfluorimetry. Cells were prepared, stained, and analyzed as described by Yen and Pardee (51).

Protein labeling and analyses. Cells in 35-mm culture dishes were labeled for 2 h with [^{35}S]methionine by incubation with 0.7 ml of methionine-free DME–4 mM glutamine–10% dialyzed serum–70 μCi of [^{35}S]methionine (600 Ci/mmol) at 37°C. The labeled cells were washed with cold phosphate-buffered saline, lysed in 100 to 200 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, and stored at −20°C. Proteins were separated on 5% acrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (26). The gels were fixed in 40% methanol and 9% acetic acid, dried, and exposed to Kodak XAR-5 film for 4 h.

Materials. Media and serum were from Flow Laboratories, Inc. Radioisotopes were from New England Nuclear Corp. or ICN Pharmaceuticals Inc. Retinoic acid, dibutyryl cyclic AMP, and theophylline were from Sigma Chemical Co., and sodium butyrate was from ICN. Restriction enzymes were from New England BioLabs, and S1 nuclease was from Boehringer Mannheim Corp.

RESULTS

Cell proliferation differentiation, and c-*myc* mRNA levels. RA induces F9 cells to differentiate into primitive endoderm (43); agents that raise intracellular cyclic AMP enhance the response and induce further differentiation to parietal endoderm (44). After addition of the inducers—RA or RA-dibutyryl cyclic AMP-theophylline (RACT)—growth continued unperturbed for at least 2 days. Thereafter, proliferation sharply declined (RA) or ceased (RACT) (Fig. 1A). Only after 3 to 4 days of incubation had greater than 95% of the cells acquired the differentiated morphology (data not shown). By then, RACT-treated cultures produced large amounts of collagen, laminin, and other differentiation-specific proteins (see Fig. 4C), in agreement with the findings of Strickland et al. (44).

The relative abundance of c-*myc* mRNA was quantitated by S1 nuclease analysis with a 5′-end-labeled probe containing murine c-*myc* exon 2 sequences (Fig. 1B). Either inducer (RA or RACT) caused a marked reduction (20- to 40-fold) in the steady-state level of c-*myc* mRNA after 72 h (Fig. 1B). Strict quantitation of these reductions was difficult because of the low level of c-*myc* mRNA present after differentiation and the variability in stem cell contamination from experiment to experiment. By contrast the relative abundance of actin mRNA was unaltered (Fig. 1B). In addition, our prior results showed that after RACT-induced differentiation c-ras^{Ha} mRNA levels decreased less than twofold, whereas c-ras^{Ki} mRNA declined about fivefold (6). Thus, RA induced a decline in c-*myc* mRNA that was selective for this proto-oncogene and was independent of added dibutyryl cyclic AMP.

RACT-treated cultures remained viable and showed no change in the low level of c-*myc* mRNA for at least 6 days. When RACT was removed from the medium after 72 h, the mRNA level did not rise, at least within an ensuing 24 h of incubation in medium lacking inducers (data not shown).

The onset of the decline in the c-*myc* mRNA level was very early and occurred after a discrete lag. RNA was isolated at 4-h intervals after the addition of RACT, and the abundance of c-*myc* mRNA sequences was quantitated by

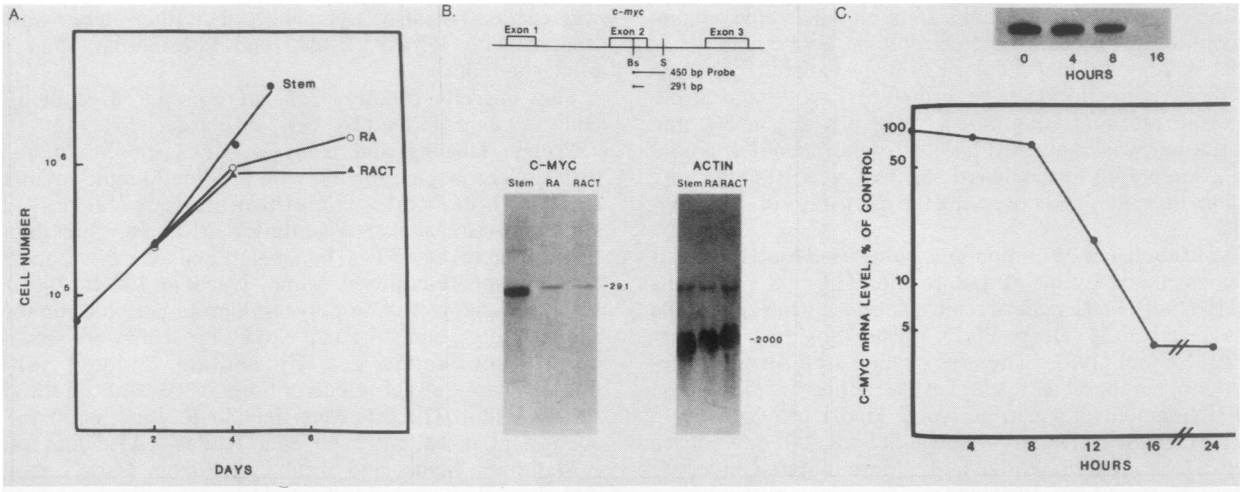

FIG. 1. Cell growth and c-*myc* mRNA levels during F9 cell differentiation. A, Time course of cell proliferation. At 24 h after plating (day 0), F9 cells were given control medium (no additions, Stem), medium supplemented with 2.5×10^{-7} M RA, or medium supplemented with 2.5×10^{-7} M RA–0.5 mM dibutyryl cyclic AMP–0.25 mM theophylline (RACT). At the indicated times thereafter, the number of cells per plate was determined. B, c-*myc* and actin mRNA after RA- or RACT-induced differentiation. Cells (10^6 per 150-mm dish) were plated and shifted to appropriate media as described above, except that the RA concentration was 10^{-6} M. After 72 h, RNA was extracted from the cells and purified. c-*myc* mRNA abundance was quantitated by S1 nuclease analysis by using a 5′ end-labeled exon 2 probe and 15 μg of RNA as described in Materials and Methods and as shown by the diagram above the RNA analyses. Bs and S are the *Bss*HII and *Sac*I restriction sites, respectively. c-*myc* mRNA quantitatively protects from S1 digestion a 291-base fragment as indicated. Actin mRNA abundance was quantitated by Northern analysis; the position of the 2-kilobase actin mRNA is indicated (5). C, Time course of c-*myc* mRNA decline. Cells were plated and shifted into RACT as described for panel B. At the indicated times, RNA was isolated and analyzed for c-*myc* mRNA by S1 nuclease analysis. The line drawing expresses the results as the percentage of c-*myc* mRNA abundance in untreated stem cells, determined by densitometry of the autoradiograms, versus time. From the semilog plot, we estimate that after 8 h, the c-*myc* mRNA level decays with a half-life of less than 2 h.

S1 analysis (Fig. 1C). The mRNA level remained nearly constant for 8 h and then abruptly fell more than 20-fold between 8 and 16 h. c-*myc* mRNA declined, then, with unique kinetics and well in advance of growth arrest.

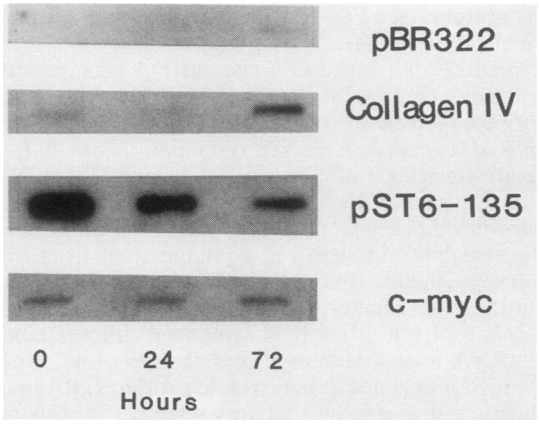

FIG. 2. c-*myc* transcription level during F9 cell differentiation. F9 cells (1×10^6 to 1.5×10^6) were plated onto 150-mm culture dishes. After 24 h, nuclei were isolated from stem cells (0 h); the remaining cells were shifted into RACT, and nuclei were isolated 24 and 72 h later. Labeled, runoff RNA was hybridized to plasmid (pBR322)-containing sequences specific for collagen IV, pST6-135, and c-*myc*. The c-*myc* clone used for this experiment contained both exons and introns. We have used this clone to probe *myc* sequences in the mouse genome, and it detects only single-copy sequences (6). Groudine and Casmir (20) have similarly shown that introns within c-*myc* do not detect repetitive sequences. In addition, this experiment was repeated, with identical results, with a cDNA clone (41).

c-*myc* expression is posttranscriptionally regulated. The decrease in c-*myc* mRNA occurred despite little or no change in the level of *myc* gene transcription. Nuclei were isolated from untreated cells and after incubation with RACT. ^{32}P-labeled runoff transcripts were isolated and hybridized to immobilized plasmid DNA (Fig. 2). Nuclei from cells before and after 24 h of incubation with RACT synthesized nearly equal amounts of RNA hybridizable to *myc* sequences, indicating that the level of transcription was essentially unaltered. After 72 h, a slight decrease (less than twofold) in *myc* transcription level was observed. By contrast, an increase in RNA hybridizable to collagen IV was readily detectable, in agreement with the findings of Wang et al. (48). In addition, transcription of a developmentally regulated stem cell gene (pST6-135) decreased, as previously reported (30). Thus, the marked decrease in c-*myc* mRNA was due to posttranscriptional regulation of this gene by RACT.

RA decreases c-*myc* mRNA during an early block to differentiation. We have shown that sodium butyrate is a potent inhibitor of an early step in the RACT-induced differentiation of F9 cells. Butyrate prevents the increase in plasminogen activator activity and induction of differentiation-specific genes (collagen IV and laminin) if added no later than the first 8 h after RACT addition (29). This drug, then, defines a critical point in the commitment to differentiate.

In contrast to its effect on differentiation-specific markers, butyrate did not inhibit the decline in c-*myc* mRNA (Fig. 3). At 24 h after RACT and butyrate were added simultaneously, the steady-state level of message was about 20-fold lower relative to the level in stem cells. This decrease was always identical in magnitude to that seen in cultures treated for 24 h with RACT only. Incubation with butyrate alone resulted in only a slight (less than twofold) reduction. None

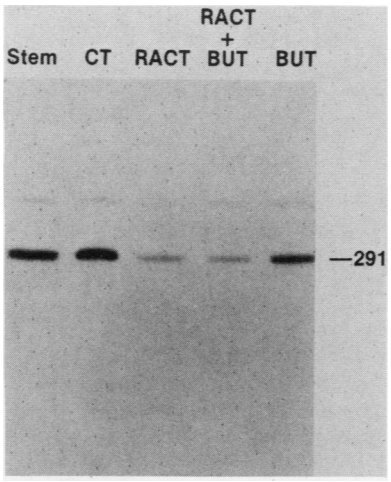

FIG. 3. c-*myc* mRNA after a differentiation block by butyrate. Cells were plated at 1.4×10^6 per 150-mm dish. After 48 h, experimental medium was added for 24 h. RNA was then isolated and analyzed for c-*myc* mRNA abundance by S1 nuclease analysis. Experimental conditions were no additions (Stem), dibutyryl cyclic AMP (0.5 mM) and theophylline (0.25 mM) (CT), RACT, RACT plus butyrate (5 mM) (RACT + BUT), and butyrate only (BUT). The position of the 291-base fragment protected by c-*myc* mRNA is indicated.

of these treatments altered cell growth within the 24-h period under study. The results show that an early block to differentiation did not prevent the RA-directed decrease in c-*myc* mRNA levels.

Figure 3 also shows that dibutyryl cyclic AMP and theophylline do not affect c-*myc* mRNA abundance; together with the data in Fig. 1B, this demonstrates that regulation of this gene expression is entirely under RA control.

***c-myc* expression declines after growth arrest without differentiation.** Embryonal carcinoma cells are highly proliferative (38) and do not arrest growth under conditions that commonly limit proliferation in most differentiated cells (e.g., serum deprivation or growth to confluence). However, F9 cells do partially arrest growth when deprived of an essential amino acid (isoleucine) for 40 to 48 h. Isoleucine starvation decreased the fraction of cells in the S phase from approximately 80% to 30% and increased proportionally the fraction of cells in G1 (Fig. 4A). In growth-arrested cells, the abundance of c-*myc* mRNA was reduced nearly 10-fold relative to unperturbed stem cells (Fig. 4B). The actin mRNA level, by contrast, did not change (data not shown). When cells were deprived of isoleucine for longer than 48 h, the c-*myc* mRNA level was not further reduced, but the cells began to die.

Differentiation-specific proteins were not synthesized after starvation for isoleucine (Fig. 4C). However, major stem cell proteins were made in normal amounts during a 2-h pulse with [^{35}S]methionine in isoleucine-containing medium given immediately after starvation. This result further indicates that major mRNA species remained intact during the deprivation period. At 24 h after isoleucine was restored, growth resumed (data not shown), and the pattern of proteins synthesized remained characteristic of stem cells (Fig. 4C). This was also true 72 h after isoleucine readdition; however, the culture had to be split after the first day to prevent overcrowding (data not shown). Thus, decreased proliferation in the absence of differentiation was accompanied by reduced c-*myc* mRNA levels.

DISCUSSION

Our results demonstrate strong, negative, posttranscriptional control over the *myc* proto-oncogene by RA at an early step in the differentiation of F9 cells. The decrease in c-*myc* mRNA level is the earliest change in specific gene expression that has been observed in this system, and it is noteworthy that the regulation was not transcriptional. The decline in mRNA abundance began 8 h after RA addition, the same time point at which F9 cells are critically sensitive to inhibition of differentiation by butyrate (29). We suggest, therefore, that determinative changes in gene expression are initiated 8 h after exposure to RA. One type of change, initially sensitive to butyrate, directs the synthesis of differentiation-specific molecules. Another, insensitive to butyrate, directs decreased expression of c-*myc* and possibly other genes.

Two experiments correlate c-*myc* mRNA levels with proliferation rather than differentiation. An early block to differentiation did not impede the decline in mRNA level, and decreased proliferation in the absence of differentiation resulted, nonetheless, in reduced expression. Alterations in c-*myc* mRNA abundance have been associated with changes in growth state in several cell systems including murine fibroblasts (6, 23), human lymphocytes (23), and rat liver (17, 33). Moreover, transfection experiments suggest a functional role for this gene in growth control (1), and loss of regulation is often associated with tumorigenicity (6, 28). In F9 cells, the decline in c-*myc* mRNA preceded the decline in proliferation by 48 to 72 h. It is unlikely that the *myc* protein persists for 48 to 72 h, since it has been shown to be very short lived (22). The function of c-*myc* in the growth of these cells, therefore, may be as a primary regulator of other genes or processes more directly involved in cell cycle progression and DNA synthesis. However, c-*myc* mRNA and protein levels were unresponsive to growth state in normal avian fibroblasts (22, 47). Thus, regulation (or perhaps function) of this gene might vary depending upon the species, cell type, or culture conditions.

It is striking that the decline in c-*myc* mRNA is effected posttranscriptionally. Posttranscriptional control of other growth-related genes—notably dihydrofolate reductase (52) and thymidine kinase (20)—has been demonstrated. Unlike c-*myc*, dihydrofolate reductase and thymidine kinase expression is more tightly linked to DNA synthesis. Transcriptional regulation of c-*myc* has been observed in some cells stimulated to grow or differentiate. In density-arrested and stimulated BALB/c 3T3 cells, a 3-fold *myc* transcriptional increase could not account for the 20- to 40-fold rise in mRNA level (18). By contrast, when HL-60 promyelocytic leukemia cells were induced to terminally differentiate, the 6- to 20-fold decrease in transcription could account for the reduced c-*myc* mRNA level (19). Also, in these cells, c-*myc* mRNA declined to a greater extent during differentiation than after growth arrest in 0.2% serum (14). HL-60 cells carry an amplified *myc* gene which may be subject to altered regulation. Alternatively, hematopoietic cells may differ from EC cells in their response to perturbations of growth or differentiation.

We do not yet know whether posttranscriptional modulation of c-*myc* in F9 cells is the result of altered processing, enhanced degradation, or both. Results in other cell systems suggest that mRNA turnover plays a role in c-*myc* regulation (11). Thus, we speculate that an early action of RA is

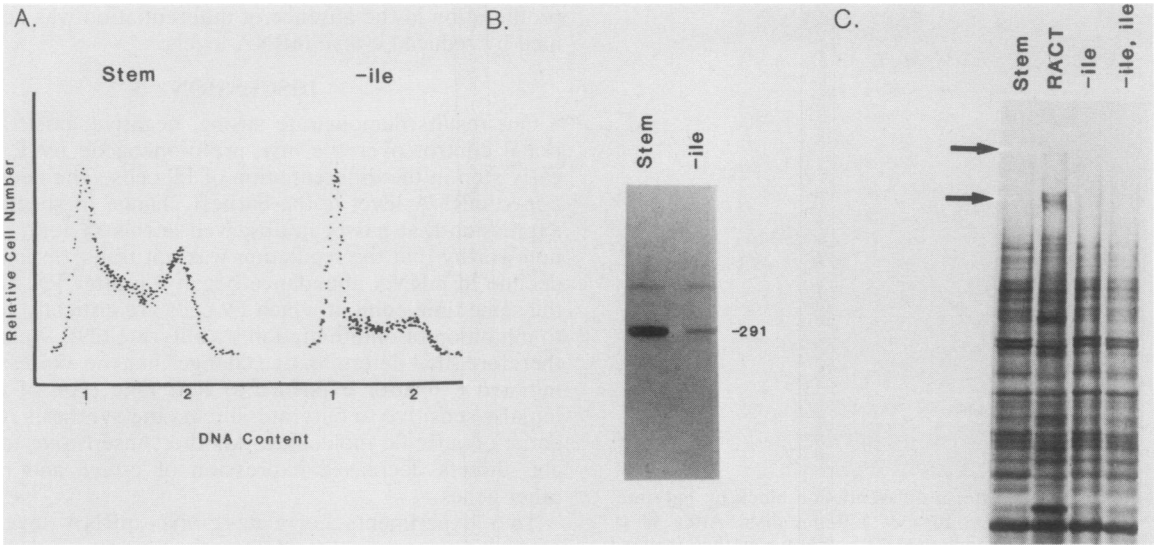

FIG. 4. Growth arrest and c-*myc* mRNA. F9 cells (3×10^{-6}) were plated on 150-mm dishes. After 24 h, the cultures were washed with isoleucine-free DME and incubated for 48 h in isoleucine-free DME and 15% dialyzed serum. A, Flow microfluorimetric analysis of DNA content of stem and isoleucine-starved (−ile) cells. The vertical axis shows relative cell number; the horizontal axis shows fluorescence or DNA content. Cells having a G1 DNA content are represented in the first and major peak; cells in G2 are represented in the second smaller peak; cells in S phase fall between the G1 and G2 peaks. B, S1 nuclease analysis of c-*myc* mRNA sequences in total RNA isolated from stem and isoleucine-starved (−ile) cells. The position of the 291-base protected fragment is indicated. C, Proteins synthesized by isoleucine-starved cells. Cells (1×10^4 for stem and RACT cultures; 3×10^4 for starved cultures) were plated onto 35-mm dishes. After 24 h, they were washed and incubated in control medium, RACT, or isoleucine-free DME and 15% dialyzed serum. After 48 h, one of the starved cultures (−ile) and the control culture (stem) were labeled with [^{35}S]methionine as described in Materials and Methods. Another starved culture was given complete medium; after an additional 24 h, the RACT and refed (−ile, ile) cultures were labeled, and 10^7 acid-precipitable counts were loaded into each lane. The most prominent differentiation-specific proteins are visible in the high-molecular-weight portion of the RACT lane. The upper arrow indicates intracellular laminin A, and the lower arrow designates the disperse band of intracellular laminin B and collagen IV (α_1 and α_2, respectively) (29, 44).

induction or activation of specific nuclease activity. This action, which is butyrate insensitive, is distinct from the induction of differentiation-specific genes, which is butyrate sensitive. Butyrate is known to modify chromosomal proteins, in particular histones (7). Although we could not rule out other actions, butyrate did inhibit histone deacetylation in F9 cells (29). Thus, alterations in chromatin composition might also be initiated as early as 8 h after RA addition.

c-*myc* mRNA levels declined with varied kinetics during terminal differentiation of several cell types (16, 25, 36). In these systems, differentiation and arrested growth were not distinguished. However, Thiele et al. (46) found that decreased expression of N-*myc*, which shares limited homology to c-*myc*, preceded growth arrest and differentiation of neuroblastoma cells. In addition, Stewart et al. (42) reported that seminiferous tubules contained low levels of c-*myc* mRNA during a developmental stage where a small but significant proportion of cells are proliferating spermatogonia. These investigators suggested that immature spermatogonia may undergo final cell divisions in the absence of *myc* transcripts. This is clearly what happens in differentiating F9 cells. Although we cannot rule out a role for *myc* in differentiation, our results suggest that growth arrest may be directed by changes in which c-*myc* plays an initiating role, and that these changes are separable from those directing other features of the differentiated phenotype.

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ADDENDUM IN PROOF

Dony et al. (C. Dony, M. Kessel, and P. Gruss, Nature [London] **317**:636–639, 1985) recently demonstrated posttranscriptional regulation of c-*myc* expression during the differentiation of F9 cells; our results and theirs are in essential agreement.

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