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Enforced Expression of the c-*myc* Oncogene Inhibits Cell Differentiation by Precluding Entry into a Distinct Predifferentiation State in G_0/G_1

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A broad base of data has implicated a role for the c-*myc* proto-oncogene in the control of the cell cycle and cell differentiation. To further define the role of *myc* in these processes, I examined the effect of enforced *myc* expression on several events that are thought to be important steps leading to the terminally differentiated state: (i) the ability to arrest growth in G_0/G_1, (ii) the ability to replicate the genome upon initiation of the differentiation program, and (iii) the ability to lose responsiveness to mitogens and withdraw from the cell cycle. 3T3-L1 preadipocyte cell lines expressing various levels of *myc* mRNA were established by transfection with a recombinant *myc* gene under the transcriptional control of the Rous sarcoma virus (RSV) promoter. Cells that expressed high constitutive levels of pRSVmyc mRNA arrested in G_0/G_1 at densities similar to those of normal cells at confluence. Upon initiation of the differentiation program, such cells traversed the cell cycle with kinetics similar to those of normal cells and subsequently arrested in G_0/G_1. Thus, enforced expression of *myc* had no effect on the ability of cells to arrest growth in G_0/G_1 or to replicate the genome upon initiation of the differentiation program. Cells were then tested for their ability to reenter the cell cycle upon exposure to high concentrations of serum and for their capacity to differentiate. In contrast to normal cells, cells expressing high constitutive levels of *myc* RNA reentered the cell cycle when challenged with 30% serum and failed to terminally differentiate. The block to differentiation could be reversed by high expression of *myc* antisense RNA, showing that the induced block was specifically due to enforced expression of pRSVmyc. These findings indicate that 3T3-L1 preadipocytes enter a specific state in G_0/G_1 after treatment with differentiation inducers, into which cells expressing high constitutive levels of *myc* RNA are precluded from entering. I propose that *myc* acts as a molecular switch and directs cells to a pathway that can lead to continued proliferation or to terminal differentiation.

A compelling body of evidence indicates that the c-*myc* proto-oncogene is involved in the control of cell proliferation. Expression of c-*myc* increases rapidly and transiently when quiescent cells reenter the cell cycle in response to stimulation by growth factors such as platelet-derived growth factor (27). Similarly, injection of purified c-*myc* protein into cell nuclei leads to the onset of DNA synthesis when cells are subsequently exposed to plasma (25). Thus, in the competence-progression model of the cell cycle, c-*myc* has been classified as a competence factor capable of stimulating cells out of a G_0/G_1 quiescent state and into S phase. Although c-*myc* mRNA levels change dramatically when quiescent cells reenter the cell cycle, c-*myc* expression is invariant throughout the cell cycle (21, 51). The findings that elevated c-*myc* expression is associated with many naturally occurring neoplasms support a role for c-*myc* in the control of cell proliferation (1, 11, 12, 34).

High constitutive expression of c-*myc* blocks the differentiation of mouse erythroleukemic cells into cells that resemble mature erythrocytes (6, 8, 43). Similarly, expression of a recombinant c-*myc* gene subjugated to the immunoglobulin heavy-chain enhancer in transgenic mice perturbs normal B-lymphocyte development (30). Animals expressing the transgene contain an abnormally expanded population of nonmalignant premature B lymphocytes in their bone marrow. These findings suggest that c-*myc* might have a role in regulating cell differentiation, as well as in controlling cell proliferation. It has been shown in several cell systems that repression of c-*myc* expression occurs concomitantly with terminal differentiation (9, 10, 15, 29, 44, 52). These observations raised the possibility that irreversible suppression of c-*myc* expression is necessary for withdrawal from the cell cycle, a condition that is a requisite for terminal differentiation. Studies with rat skeletal myoblasts indicate that this is not the case. The c-*myc* gene is inducible in both biochemically and terminally differentiated myotubes, and transient induction of c-*myc* does not suppress the differentiated phenotype (10). However, these studies do not exclude the possibility that a transient suppression of c-*myc* gene expression, at a specific point during the differentiation program, is a necessary event for terminal differentiation. The biphasic pattern of c-*myc* gene expression observed in several differentiating cell systems supports the hypothesis that a transient reduction in c-*myc* RNA levels may be necessary for irreversible withdrawal from the cell cycle (9, 10, 29, 52).

It is has long been thought that cell proliferation and cell differentiation are coupled but exclusive events. This hypothesis is based on the observation that cessation of proliferation is usually associated with terminal differentiation and that many terminally differentiated cell types are unable to reenter the cell cycle when challenged with mitogens (17, 40, 41). Although attractive as this hypothesis might be, many malignant tumors are capable of acquiring a highly differentiated phenotype. Given the likely role of c-*myc* in these cellular processes, I examined the effect of enforced c-*myc* expression on several events that are thought to represent

important steps leading to the terminally differentiated state. The results indicate that enforced expression of c-*myc* precludes cells from entering into a specific growth-arrested state in G_0/G_1 that is a requisite for terminal differentiation.

MATERIALS AND METHODS

Construction of *myc* genes. pRSVmyc was constructed as follows. A 1.9-kilobase (kb) restriction fragment containing c-*myc* cDNA sequences was excised from pMc myc-54 (49) by digestion with *Hin*dIII. The ends were made blunt with the large fragment of *Escherichia coli* DNA polymerase I, and the fragment was cloned into the *Hin*cII site of pUC-8. The c-*myc* sequences were then excised as a 1.9-kb *Hin*dIII-*Bam*HI fragment and inserted between the *Hin*dIII and *Bgl*II sites of RSV β-globin (16) after removal of the β-globin sequences. pSV2 myc^{as}-dhfr was provided by E. Prochownik (University of Michigan) and was constructed in a fashion previously described except that c-*myc* cDNA sequences were cloned in the antisense orientation (43).

Cell culture. Cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 2 mM glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) 100 μg of streptomycin sulfate per ml, and 100 U of penicillin G per ml (growth medium) in an atmosphere of 90% air and 10% CO_2. To initiate the differentiation program, confluent monolayers (5×10^4 cells per cm^2) were treated with growth medium supplemented with 0.25 μM dexamethasone, 0.5 mM methylisobutylxanthine, and 10 μg of bovine insulin per ml (differentiation medium) for 24 to 48 h. The medium was changed, and thereafter the cells were sustained in growth medium. The medium was changed every 3 days. 3T3-L1 cells were obtained from the American Type Culture Collection, Rockville, Md.

DNA transfections. Cells were plated at an approximate density of 10^4 cells per cm^2 1 day before transfection. Transfections were performed by the $CaPO_4$-DNA precipitation method (42). Supercoiled plasmid DNA was used unless indicated otherwise. A 5:1 molar ratio of the nonselectable gene to the selectable gene was used in all transfections. The day after the addition of the $CaPO_4$-DNA precipitate to monolayers, cells were subjected to selection in growth medium containing either 0.4 mg of hygromycin B (Calbiochem-Behring, La Jolla, Calif.) per ml or 1.0 mg of G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml, depending on whether the hygromycin or neomycin resistance marker was used. Colonies were isolated 2 weeks later by using cloning cylinders, and cell lines were maintained in selective medium for an additional 2 weeks. Mas cell lines were generated by cotransfection of Myc-1 cells (containing the hygromycin resistance marker) with pSV2 myc^{as}-dhfr and pRSVneo plasmid DNA followed by selection in growth medium containing G418. The pSV2 myc^{as}-dhfr plasmid was linearized with *Nde*I before transfection to increase the likelihood that *myc* antisense gene sequences and *dhfr* gene sequences would remain linked upon integration. Cloned cell lines were established, and cells were then subjected to selection in Dulbecco modified Eagle medium containing 10% dialyzed fetal calf serum and 0.25 μM methotrexate. Amplification of *myc* antisense gene sequences was obtained by successive selections with fourfold-increased concentrations of methotrexate. The cells were grown in each selective medium for 1 month before being transferred to the next.

Analysis of DNA and RNA. Genomic DNA was isolated and subjected to Southern blot analysis as previously described (14). RNA was isolated by lysing cells in guanidinium isothiocyanate followed by centrifugation in CsCl density gradients (37). S1 nuclease assays were performed as described previously (37), and DNA probes were labeled with [γ^{32}P]ATP by standard procedures (37). RNase A protection experiments were performed by published procedures (39) except that samples were treated only with 40 μg of RNase A per ml. Nuclease-resistant fragments were applied to 8% polyacrylamide sequencing gels, and radioactive bands were visualized by autoradiography. [^{32}P]RNA probes were generated from DNA templates which contained c-*myc* cDNA sequences inserted into the Gem 1 plasmid. The RNA probes (specific activities, 0.5×10^9 to 2×10^9 cpm/μg of DNA template) were generated by using SP6 RNA polymerase of T7 RNA polymerase (39), depending on which RNA strand needed to be labeled. For Northern blot (RNA blot) analysis, cytoplasmic RNA was isolated, denatured with glyoxal, and subjected to electrophoresis in 1% agarose (37). A portion of the gel was stained with ethidium bromide to visualize the rRNA bands. The RNA was transferred to nitrocellulose and prehybridized in 50% formamide–1 M NaCl–50 mM $NaPO_4$–10 mM EDTA–0.2 g each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll 400 (Pharmacia Fine Chemicals, Piscataway, N.J.) per liter–0.25 mg of denatured salmon sperm DNA per ml–10% (wt/vol) dextran sulfate–1% sodium dodecyl sulfate (SDS) at 58°C for 12 h; 10^7 cpm of a mouse 18S rRNA-cDNA probe and 10^8 cpm of the RNA probe were added, and hybridization proceeded at 58°C for 24 h. The blot was washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS at room temperature, twice in 0.1× SSC–0.5% SDS at 60°C, twice in 2× SSC without SDS at room temperature, twice in 2× SSC containing 10 μg of RNase A per ml at room temperature for 15 min, and once in 0.1× SSC–0.5% SDS at 60°C. Treatment with RNase A was necessary to remove nonspecific hybridization to the rRNAs.

[^{3}H]thymidine incorporation. Cells were plated into 16-mm-diameter microdilution wells and grown to confluence in growth medium. Confluent monolayers were treated with differentiation medium for 48 h, during which cells were pulsed every 3 h with 5 μCi of [*methyl*-^{3}H]thymidine (Amersham Corp., Arlington Heights, Ill.; 43 Ci/mmol; 10 μCi/ml) for 30 min. Cells were lysed in 0.1 N NaOH–10 mM EDTA–0.5% SDS and incubated at 70°C for 30 min to hydrolyze RNA. Samples were cooled to room temperature, and radioactive nucleic acid was precipitated in cold 5% trichloroacetic acid at 4°C. Radioactive nucleic acid was collected on Whatman GF/C filters (Whatman, Inc., Clifton, N.J.). The filters were washed thoroughly with 5% trichloroacetic acid and then with 95% ethanol and dried, and the radioactivity was counted.

Flow microfluorimetry. Cells were harvested by trypsinization and washed once with phosphate-buffered saline (pH 7.4). The cells were resuspended in 150 μl of phosphate-buffered saline, 350 μl of a solution containing 10 mM Tris hydrochloride (pH 8.0), 10 mM NaCl, 0.1% Nonidet P-40, 10 μg of RNase A per ml, and 7×10^{-5} M propidium iodide was added, and the cells were incubated on ice in the dark until analyzed (within 2 h). Fifteen thousand cells were analyzed in all samples with a Coulter Epics V flow cytometer (EPICS Division, Coulter Electronics, Inc., Hialeah, Fla.). The percentage of cells in each phase of the cell cycle was determined by integration of each peak within two standard deviations of the peak height by using the EASY analysis system and the PARA I program.

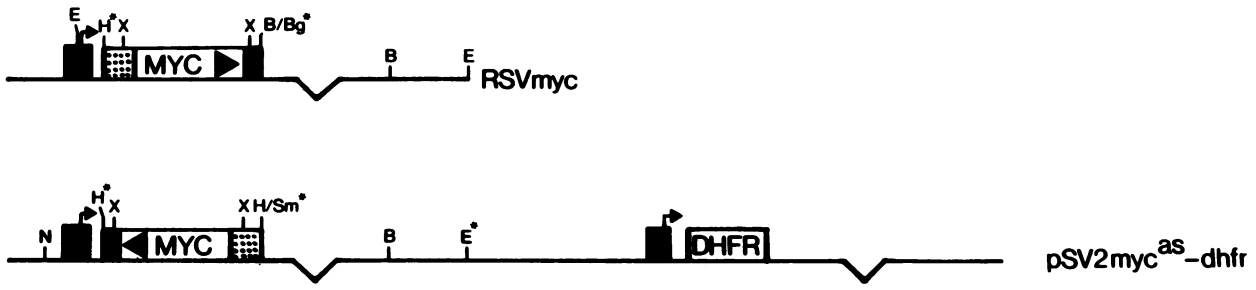

FIG. 1. Construction of pRSVmyc and pSV2 myc^{as}-dhfr plasmids. The large solid boxes with the arrows represent Rous sarcoma virus long terminal repeat (pRSVmyc) or simian virus 40 early promoter sequences (pSV2 myc^{as}-dhfr), and the arrow indicates the transcription initiation site. Stippled region, *myc* 5′ untranslated sequences; open box, *myc* coding sequences with the orientation indicated by the arrowhead; small solid box, *myc* 3′ untranslated sequences; thin line, vector sequences derived from the RSV β-globin gene. The asterisk indicates that the site was destroyed or modified during the construction. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; N, *Nde*I; Sm, *Sma*I; X, *Xho*I.

RESULTS

Construction of 3T3-L1 cell lines expressing high constitutive levels of *myc* RNA. I used the murine embryonic cell line 3T3-L1 as a model system to study cell differentiation (17). In the undifferentiated state, 3T3-L1 cells have morphological and biochemical properties resembling those of fibroblasts. Such cells synthesize large amounts of type I collagen and have little lipogenic capacity. When treated with lipogenic agents such as insulin or glucocorticoid hormones or both, 3T3-L1 cells differentiate into cells that resemble mature mammalian adipocytes. The differentiation process is accompanied by a large increase in cytosolic triglyceride and increases in many enzymatic activities involved in triglyceride biosynthesis (2, 17, 18, 36, 48). 3T3-L1 cells expressing various levels of *myc* RNA were obtained by transfection with the two recombinant plasmids shown in Fig. 1. pRSVmyc contains a mouse c-*myc* cDNA under the transcriptional control of the Rous sarcoma virus long terminal repeat. This construct contains all of the c-*myc* coding sequences, as well as portions of the 5′ and 3′ untranslated regions, and therefore produces a normal c-*myc* protein. In pSV2 myc^{as}-dhfr, the mouse c-*myc* cDNA is positioned downstream of the simian virus 40 early promoter and in the antisense orientation. The *myc* antisense gene is linked to a mutant mouse *dhfr* cDNA which allows for coamplification of these sequences by methotrexate selection (46). 3T3-L1 cells containing stably integrated copies of pRSVmyc were obtained by cotransfection with plasmids which confer resistance to the aminoglycoside antibiotic G418 or hygromycin B (16). Cloned cell lines were screened for expression of pRSVmyc by S1 nuclease mapping; the results for four cell lines are shown in Fig. 2a. The cell line designated Myc-1 expressed the highest level of pRSVmyc mRNA, approximately 40 to 50 times the level of c-*myc* mRNA produced in density-arrested 3T3-L1 cells (see below), and was used in the remaining experiments.

A time course of expression of the endogenous c-*myc* mRNA and pRSVmyc mRNA in Myc-1 cells after initiation of the differentiation program is shown in Fig. 2b. The c-*myc* gene was expressed at a very low level in density-arrested Myc-1 cells, and expression increased rapidly and transiently upon addition of fresh differentiation medium. Expression peaked after 2 h at a level at least 50 times that for density-arrested cells and gradually declined thereafter. The rapid and transient induction of c-*myc* mRNA in Myc-1 cells was identical to that observed in normal 3T3-L1 cells (data not shown) and BALB/c 3T3 cells after stimulation with serum or growth factors belonging to the competence class (7, 27). In contrast, expression of pRSVmyc mRNA was constitutive throughout the entire period examined and was at a level 40 to 50 times that of the c-*myc* mRNA in density-arrested cells (Fig. 2b, 0 h) and similar to that in exponentially growing cells (data not shown). The observation that expression of pRSVmyc was constitutive and not inducible suggests that sequences necessary for induction by serum are not contained in pRSVmyc. This is consistent with previous findings indicating that serum-induced expression of the c-*myc* gene is regulated largely at the transcriptional level (7, 19), although posttranscriptional regulation

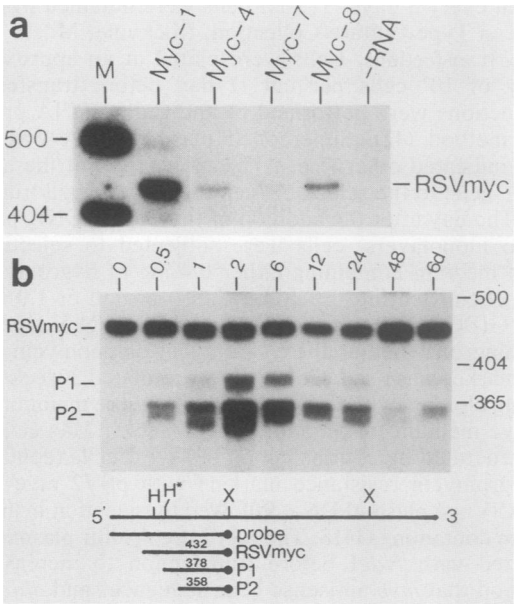

FIG. 2. (a) Quantification of pRSVmyc mRNA in transfected cell lines. Twenty-five micrograms of total RNA was used in each assay. M, Markers. (b) Quantification of c-*myc* mRNA and pRSVmyc mRNA in Myc-1 cells after addition of differentiation medium. P1 and P2 designate the two RNAs transcribed from the dual promoters of the endogenous c-*myc* gene (50). The numbers above the lanes represent hours after the addition of differentiation medium, except 6 d, which indicates days. Fifty micrograms of total RNA was used in each assay. The probe used in all assays was a 4.75-kb *Xho*I restriction fragment of pRSVmyc 5′ end labeled at the *Xho*I sites. H, *Hin*dIII; H* the destroyed *Hin*cII-*Hin*dIII site in pRSVmyc; X, *Xho*I.

has also been implicated (3). High constitutive expression of pRSVmyc did not repress expression (data not shown) or inhibit induction of the c-*myc* gene (Fig. 2b). Thus, the c-*myc* gene is not subject to negative autoregulation in 3T3-L1 cells. This is consistent with observations made with mouse erythroleukemic cells (6) and contrasts with those made with B lymphocytes (35).

High constitutive expression of *myc* blocks 3T3-L1 cell differentiation. Microscopic examination showed that treatment of 3T3-L1 cells with agents that promote differentiation resulted in the massive accumulation of cytosolic triglyceride (Fig. 3a and b). In contrast, Myc-1 cells failed to accumulate lipid when treated under identical conditions (Fig. 3c and d). Similar results were obtained with four other cell lines expressing *myc* mRNA at levels comparable to those expressed in Myc-1 cells (data not shown). Other cell lines that expressed much lower levels of pRSVmyc mRNA compared with those expressed in Myc-1 cells (Fig. 2a) differentiated into adipocytes with slightly reduced efficiencies (30 to 50%) compared with that of the parental 3T3-L1 cell line. 3T3-L1 cells transfected only with plasmids containing antibiotic resistance markers differentiated normally (data not shown). These findings indicate that a constitutive level of c-*myc* expression similar to that of cells during exponential growth is sufficient to fully inhibit 3T3-L1 cell differentiation.

The failure of Myc-1 cells to express the differentiated phenotype was not due to acquisition of a transformed phenotype. Myc-1 cells formed colonies in soft agarose with low efficiencies (less than 1%) and did not produce tumors when injected into immunosuppressed *nu/nu* mice (data not shown). There have been similar findings with mouse C3H/10$T_{1/2}$ cells expressing high levels of c-*myc* RNA (47). It is also unlikely that the observed inhibition of 3T3-L1 cell differentiation was mediated through an autocrine mechanism due to increased production of type β transforming growth factor (TGF-β), a potent inhibitor of adipocyte and myogenic cell differentiation (22, 38). I base this statement on the finding that expression of pro-α 2(I) collagen mRNA is repressed 5- to 10- fold in Myc-1 cells compared with that in normal 3T3-L1 cells (S. O. Freytag and R. J. Pogulis, submitted for publication). It was previously shown that TGF-β induces expression of pro-α 2(I) collagen, as well as other extracellular matrix proteins, in 3T3-L1 cells (23). Furthermore, cocultivation of 3T3-L1 and Myc-1 cells or treatment of 3T3-L1 cells with Myc-1 cell-conditioned medium did not significantly suppress the accumulation of differentiated cells (data not shown).

The inability of Myc-1 cells to express the differentiated phenotype was further substantiated by quantifying expression of a differentiation-specific marker, the pyruvate carboxylase gene. Expression of this gene is induced approximately 20-fold upon conversion of 3T3-L1 cells into adipocytes (14). S1 nuclease analysis showed that the concentration of pyruvate carboxylase mRNA increased more than 10-fold upon differentiation of 3T3-L1 cells (Fig. 4). In

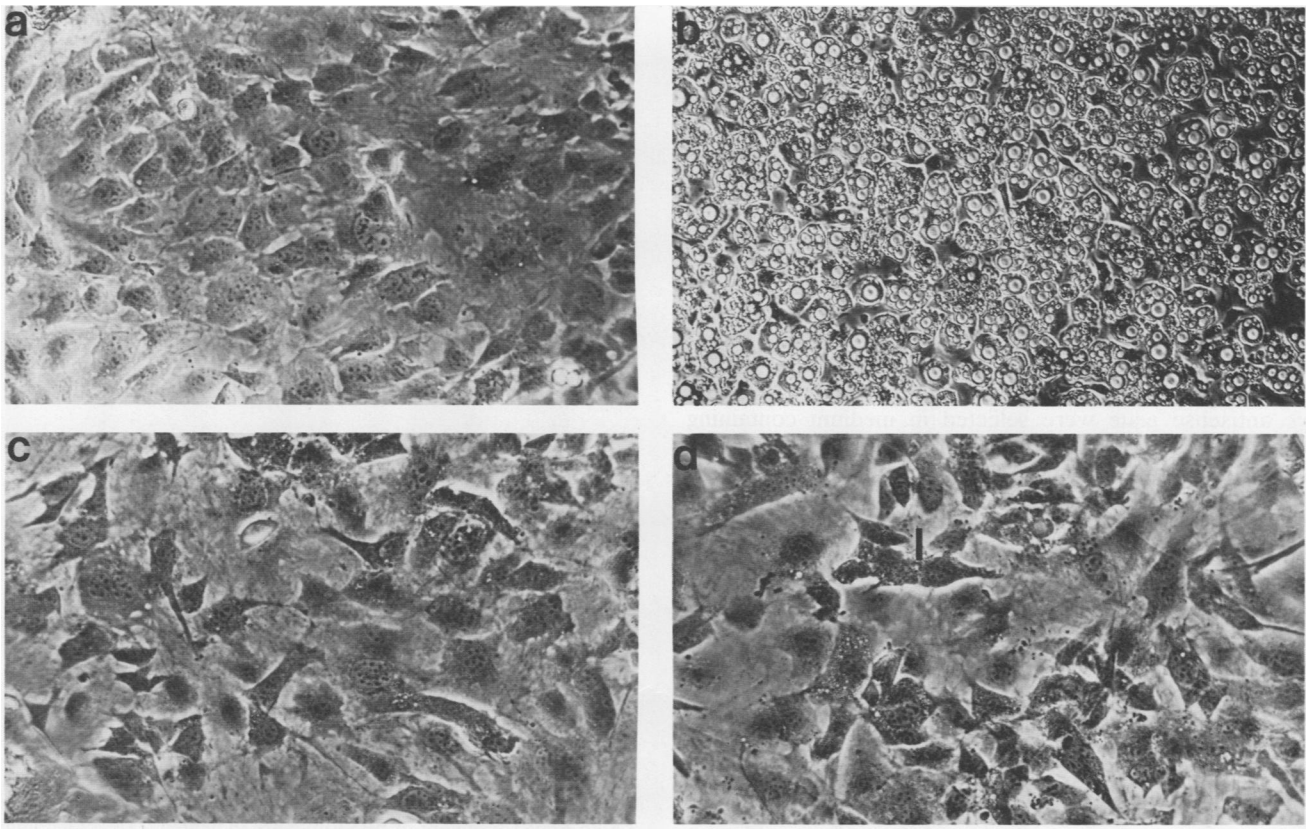

FIG. 3. Morphology of undifferentiated and differentiated 3T3-L1 and Myc-1 cells. Confluent monolayers were treated either with differentiation medium for 24 h and then sustained in growth medium supplemented with 10 μg of insulin per ml (b and d) or sustained in unsupplemented growth medium (a and c). Cells were photographed 7 days postconfluence by using phase-contrast optics at a magnification of ×160. Undifferentiated 3T3-L1 cells; (b) differentiated 3T3-L1 cells; (c) Myc-1 cells sustained in unsupplemented growth medium; (d) Myc-1 cells treated with differentiation medium.

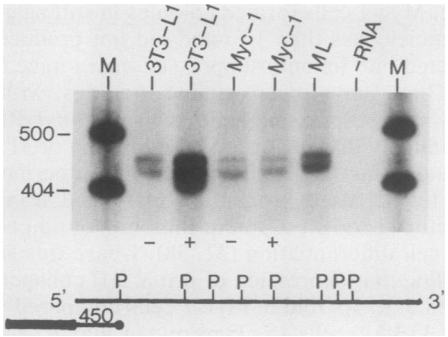

FIG. 4. Quantification of pyruvate carboxylase mRNA levels by S1 nuclease analysis. Cells were grown to confluence and induced to differentiate as described in the text. Cells were harvested 7 days after initiation of the differentiation program, and RNA was prepared. Twenty five micrograms of total RNA was used in each assay. −, Confluent monolayers sustained in unsupplemented growth medium; (+), confluent monolayers treated with differentiation medium. The probe, pmPC10, was a 3.9-kb *Pst*I restriction fragment of the mouse pyruvate carboxylase cDNA that was 5′ end labeled at a *Pst*I site located approximately 450 base pairs from the 5′ terminus of the mRNA (B.-S. Yang and S. Freytag, unpublished data). A partial restriction map of the mouse pyruvate carboxylase cDNA showing only *Pst*I sites is shown below the autoradiogram. Thin line, cDNA sequences; thick line plasmid sequences; ML, mouse liver RNA. The two protected bands are likely due to alternative mRNA splicing or multiple promoters. M, Markers.

contrast, no induction was observed in Myc-1 cells. Thus, 3T3-L1 cells expressing high constitutive levels of c-*myc* RNA are unable to express the morphological or biochemical characteristics of mature differentiated adipocytes.

Reversal of the differentiation block by *myc* antisense RNA. If the inhibition of Myc-1 cell differentiation is specifically due to enforced expression of pRSVmyc and not to nonspecific effects, it should be possible to rescue such cells from the differentiation block by decreasing pRSVmyc mRNA levels. This idea was tested by attempting to reduce pRSVmyc mRNA levels by using a gene that produced *myc* antisense RNA. This strategy has been successfully used to inhibit expression of transfected genes and endogenous cellular genes in a wide variety of cell types (24, 28). Myc-1 cells were cotransfected with pSV2 myc^{as}-dhfr and pRSV-neo, and cloned cell lines containing integrated copies of the *myc* antisense gene were selected in medium containing G418. An example of a Southern blot in which genomic DNA from several cloned cell lines was digested with *Eco*RI and probed with a restriction fragment of the mouse *myc* cDNA is shown in Fig. 5. The 3T3-L1 cell line yields one hybridizing fragment of 22 kb that represents the endogenous c-*myc* gene (26) and served as an internal control for the amount of DNA transferred to the nitrocellulose filter. Myc-1 cells contain one additional prominent band of 3.6 kb that represents pRSVmyc sequences (Fig. 1) and a minor band of 3.4 kb that probably represents a junction fragment. All cloned *myc* antisense cell lines (Mas) contain multiple copies of the *myc* antisense gene (15 to 200 copies), as demonstrated by the presence of hybridizing fragments in these cell lines that were not present in Myc-1 cells. Selecting cells in increasing concentrations of methotrexate resulted in modest amplification of several of the integrated *myc* antisense genes (Fig. 5). In Mas-3 cells, five bands were amplified three- to fivefold (closed arrowheads), whereas the intensities of the other bands remained unchanged (open arrowheads).

Cloned Mas cell lines were examined for expression of the *myc* antisense gene by using RNase A protection assays (Fig. 6a). The probe used in these assays is single stranded and complementary to *myc* antisense RNA; therefore, pRSVmyc RNA contained in these cell lines will not give protection. Abundant expression of *myc* antisense RNA was evident in most but not all of the cloned cell lines examined. In the example shown, cell lines Mas-3, Mas-7, and Mas-12 expressed the *myc* antisense RNA in readily detectable amounts, whereas a very low level of expression was observed in Mas-23 cells (evident in overexposures of the autoradiogram). As expected, RNA from 3T3-L1 and Myc-1 cells failed to give protection of the RNA probe. The ratio of *myc* antisense RNA to *myc* sense RNA in several of the Mas cell lines was estimated by Northern blot analysis with strand-specific RNA probes. The results indicate that the ratio is 20 to 50 to 1 in Mas-3 cells resistant to 16 μM methotrexate (data not shown).

It was previously shown that antisense RNA inhibits transport of RNA from the nucleus to the cytoplasm in eucaryotic cells (28). Therefore, the level of *myc* sense RNA in the cytoplasmic poly(A) mRNA fraction was quantified by Northern blot analysis (Fig. 6b). The Mas cell lines contain both *myc* sense and antisense RNA; therefore, it was necessary to use a strand-specific probe complementary to *myc* sense RNA transcripts. Since the *myc* antisense RNA contained in the Mas cell lines is immobilized after transfer to the nitrocellulose filter, it cannot compete with the [^{32}P]RNA probe for *myc* sense RNA transcripts. Therefore, any decrease in hybridization signal is due to a decrease in the concentration of *myc* sense mRNA and not to increased

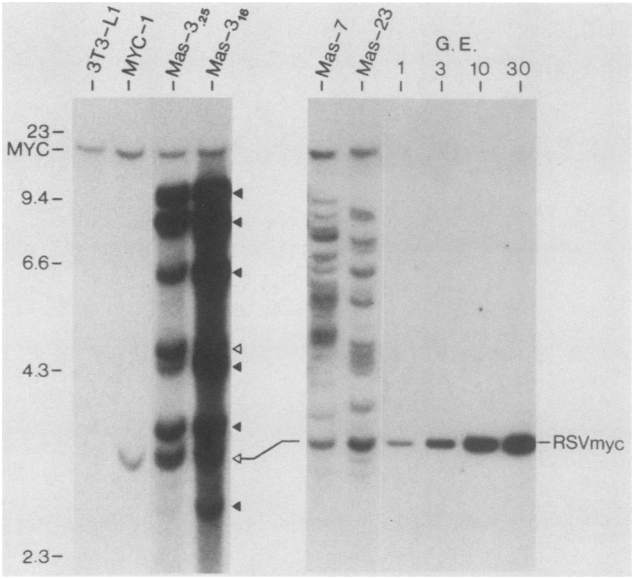

FIG. 5. Southern blot of genomic DNA from cell lines. Five micrograms of genomic DNA was digested with *Eco*RI and subjected to Southern blot analysis. The probe was a 1.4-kb *Xho*I restriction fragment of pRSVmyc and contained only *myc* cDNA sequences. ▲, Amplified bands; △, bands that were not amplified. The bands representing the endogenous c-*myc* gene (MYC) and the 3.6-kb *Eco*RI fragment of pRSVmyc are indicated. G.E., Genomic equivalents of pRSVmyc plasmid DNA digested with *Eco*RI (one genomic equivalent equals 10 picograms of pRSVmyc plasmid DNA [6 kb]). The numbers on the left represent sizes of DNA markers in kilobase pairs, and Mas-3 subscripts indicate the levels of methotrexate resistance in micromolar concentrations.

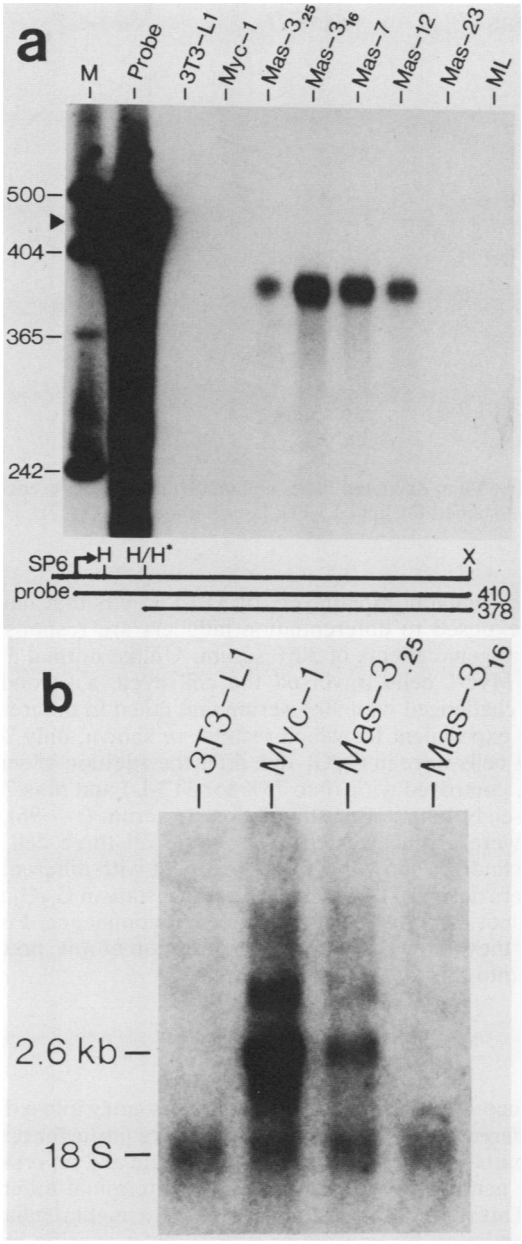

FIG. 6. (a) RNase A protection assays quantifying *myc* antisense RNA transcripts. Five micrograms of total RNA was used in all assays. Probe, 10^6 cpm of the 410-base *myc* sense RNA probe; ML, mouse liver RNA; H, *Hin*dIII; H/H*, the *Hin*cII-*Hin*dIII sites in pRSVmyc that were destroyed during its construction; X, *Xho*I. The Mas-3 subscripts indicate the levels of methotrexate resistance in micromolar concentrations. The protected fragment length is 378 bases. (b) Northern blot quantifying *myc* sense RNA transcripts. Five micrograms of cytoplasmic poly(A) mRNA was applied to each lane. The blot was hybridized with a ^{32}P-labeled *myc* antisense RNA probe and a ^{32}P-labeled cDNA probe for the mouse 18S rRNA. The latter probe was used to detect the low level of 18S rRNA in the poly(A) mRNA preparations and served as an internal control. The Mas-3 subscripts indicate the levels of methotrexate resistance in micromolar concentrations.

competition between the [^{32}P]RNA probe and *myc* antisense RNA. Consistent with previous reports (5, 7, 27) and with the S1 nuclease assays (Fig. 2), the level of c-*myc* mRNA in density-arrested fibroblasts was very low and was not detected in this analysis (Fig. 6b). In contrast, pRSVmyc mRNA (2.6 kb) was readily detected in Myc-1 cells. The steady-state concentration of pRSVmyc mRNA was significantly lower in cells expressing the *myc* antisense gene than in Myc-1 cells. In the example shown, Mas-3 cells resistant to 0.25 μM methotrexate expressed the pRSVmyc mRNA at approximately 10 to 20% of the level expressed by Myc-1 cells. Cells that were resistant to 16 μM methotrexate and contained increased amounts of *myc* antisense RNA (Fig. 6a) had undetectable levels of *myc* mRNA, similar to normal 3T3-L1 cells. Thus, expression of *myc* antisense RNA in Myc-1 cells effectively reduces the steady-state level of mature polyadenylated pRSVmyc mRNA in the cytoplasm.

Attempts were made to measure c-*myc* protein levels in these cell lines by immunoprecipitation using two different *myc*-specific sera (13, 20). However, these attempts were repeatedly unsuccessful. This failure could have been due to the fact that the c-*myc* protein is in very low abundance in density-arrested fibroblasts (20) and may be below detection in the cell lines used in these experiments. However, given the findings that cytoplasmic pRSVmyc mRNA levels were significantly reduced in Mas cell lines expressing high levels of *myc* antisense RNA (Fig. 6b) and that the c-*myc* protein has a very short half-life (21), it is very likely that c-*myc* protein levels are also reduced in the Mas cell lines compared with those in Myc-1 cells.

The Mas cell lines were tested for their ability to overcome the block to differentiation. The results clearly show that cells expressing the *myc* antisense gene regained their ability to express the differentiated phenotype (Fig. 7). Cell lines Mas-3 and Mas-7, which expressed abundant levels of *myc* antisense RNA (Fig. 6a), accumulated significant amounts of triglyceride when treated with differentiation medium. In contrast, Mas-23 cells, which expressed very low levels of *myc* antisense RNA, failed to differentiate. This shows that reversal to the normal 3T3-L1 cell phenotype was not due to a nonspecific effect of methotrexate selections. The percentage of cells that differentiated correlated with the level of *myc* antisense RNA. These data are consistent with the finding that amplification of *myc* antisense gene sequences results in reduced steady-state levels of pRSVmyc mRNA. Thus, cells expressing abundant levels of *myc* antisense RNA revert back to the original 3T3-L1 cell phenotype, as shown by their ability to express both the morphological and biochemical properties of adipocytes. These experiments provided compelling evidence that the inhibition of adipogenesis is specifically due to enforced expression of pRSVmyc.

Cells expressing high levels of *myc* RNA do not lose their responsiveness to the mitogenic effects of serum. Enforced expression of c-*myc* could block cell differentiation by perturbing one or several events that are important steps leading to the terminally differentiated state. This idea was tested by attempting to uncover a difference in the potential of cells expressing various levels of c-*myc* RNA to arrest in G_0/G_1, to replicate their genome upon initiation of the differentiation program, and to lose responsiveness to the mitogenic effects of serum. 3T3-L1, Myc-1, and Mas-7 cells were grown to confluence and exposed to differentiation medium for 48 h to initiate the differentiation program. During this period, the cells were pulsed with [^{3}H]thymidine at regular intervals to monitor the progression of cells into and through S phase. All three cell lines arrested their growth at confluence, as demonstrated by the low level of DNA synthesis (Fig. 8a, $t = 0$) and quantification of cell number as a function of time (data not shown). The low level

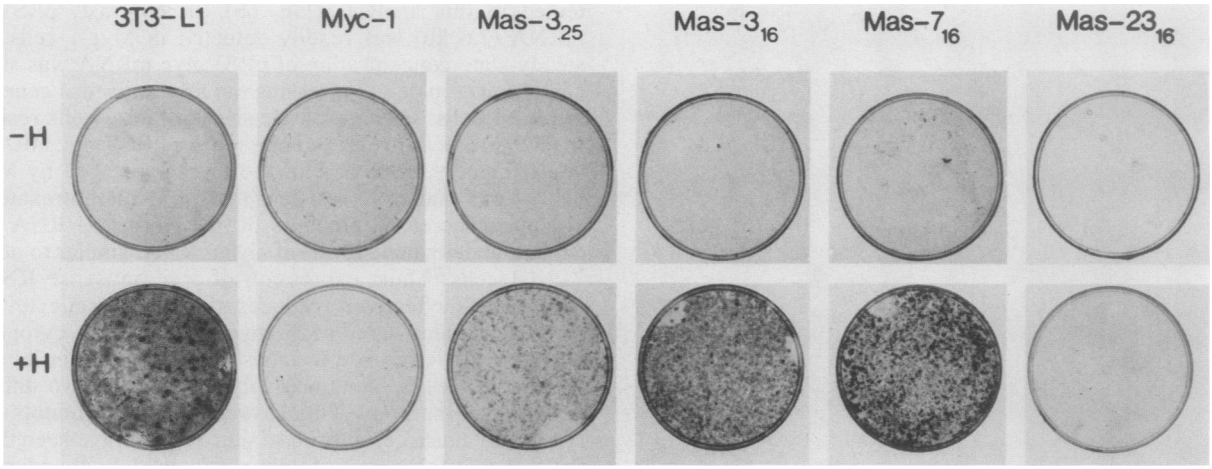

FIG. 7. Reversal of block to differentiation. Cells were grown to confluence in 60-mm-diameter dishes and incubated in the presence (+H) or absence (−H) of differentiation medium, as described in the text. Cells were stained for lipid 1 week later with oil red O (17).

of DNA synthesis was maintained for at least 7 days in cells not treated with differentiation medium (data not shown). Flow microfluorimetry analysis showed that 70 to 80% of cells arrested in G_0/G_1 at confluence in all cell lines (see below). Myc-1 cells arrested at a density of 5×10^4 to 8×10^4 cells per cm^2, which is similar to that of 3T3-L1 cells; however, Myc-1 cell monolayers attained higher densities (1.5×10^5 cells per cm^2) only after daily medium changes. This observation is similar to findings made with NIH 3T3 cells expressing high constitutive levels of a simian virus 40 *myc* gene (26).

All cell lines entered S phase 12 h after the addition of differentiation medium and progressed through S phase with similar kinetics (Fig. 8a). Depending upon the experiment, DNA synthesis reached a maximum between 18 and 21 h in all cell lines examined. Similar results were obtained when confluent cell monolayers were treated with 30% serum (data not shown). Myc-1 cells incorporated [^{3}H]thymidine at approximately two times the rate of 3T3-L1 cells when DNA synthesis peaked. This is consistent with the observation that a higher percentage of Myc-1 cells traverse the cell cycle upon addition of differentiation medium than do 3T3-L1 and Mas cells (unpublished observations), although other explanations could account for such a result. Thus, enforced expression of c-*myc* does not preclude cells from arresting their growth in G_0/G_1 or significantly alter their ability to replicate the genome upon the addition of differentiation inducers.

Forty-eight hours after initiation of the differentiation program, DNA synthesis was low (Fig. 8a) and most cells (70 to 80%) were again arrested in G_0/G_1 in all cell lines (Fig. 8b). This distribution was identical to that of cells maintained at confluence. The cells were then challenged with 30% serum, and the ability of cells to traverse the cell cycle a second time was monitored by flow microfluorimetry (Fig. 8b). The results of these experiments are striking. 3T3-L1 cells and cells expressing the *myc* antisense gene were essentially unresponsive to stimulation by serum. Greater than 70% of these cells remained in G_0/G_1 over the next 48 h, and greater than 50% differentiated into adipocytes. Both cell lines exhibited only a slightly broadening of the G_0/G_1 peak after stimulation by serum. These observations contrast with those made with normal 3T3-L1 cells arrested at confluence before treatment with differentiation inducers. As mentioned above, confluent monolayers of 3T3-L1 cells that have not been exposed to differentiation inducers are responsive to the mitogenic effects of 30% serum. Unlike normal 3T3-L1 cells, Myc-1 cells traversed the cell cycle a second time when challenged with 30% serum and failed to differentiate. In the experiment for which results are shown, only 22% of Myc-1 cells were in G_0/G_1 18 h after the addition of serum (t = 66), compared with 70 to 75% for 3T3-L1 and Mas-7 cells. Forty-eight hours after stimulation by serum (t = 96), most cells were again arrested in G_0/G_1 in all three cell lines. These findings show that after treatment with differentiation inducers normal 3T3-L1 cells arrest in a state in G_0/G_1 which is distinct from that of cells arrested at confluence. Furthermore, they show that enforced expression of *myc* precludes entry into this state.

DISCUSSION

Enforced expression of c-*myc* precludes entry into a distinct predifferentiation state in G_0/G_1 that is a requisite for terminal differentiation. Entry into a quiescent state and repression of c-*myc* gene expression often precedes terminal differentiation. This raised the possibility that c-*myc* might regulate cell differentiation by controlling entry into a specific growth-arrested state in G_0/G_1 that is a requisite for terminal differentiation. Entry into this state could represent the initiation of commitment in which differentiation will ensue without further exposure to inducers. One goal of these experiments was to uncover a difference in the ability of cell lines expressing various levels of c-*myc* RNA to carry out steps that are potentially important events leading to the terminally differentiated state. These events are the ability to arrest in G_0/G_1, to replicate the genome shortly after initiation of the differentiation program, and to lose responsiveness to mitogens and withdraw from the cell cycle. All cell lines examined in this study arrested in G_0/G_1 at confluence. Thus, the high level of *myc* expression in Myc-1 cells is not sufficient to induce uncontrolled proliferation and does not alter the cell cycle distribution of cells at confluence. Confluent monolayers of Myc-1 cells will progress into S phase after stimulation by serum but not in response to platelet-poor plasma (unpublished observations). These findings indicate that in addition to high c-*myc* expression, other events

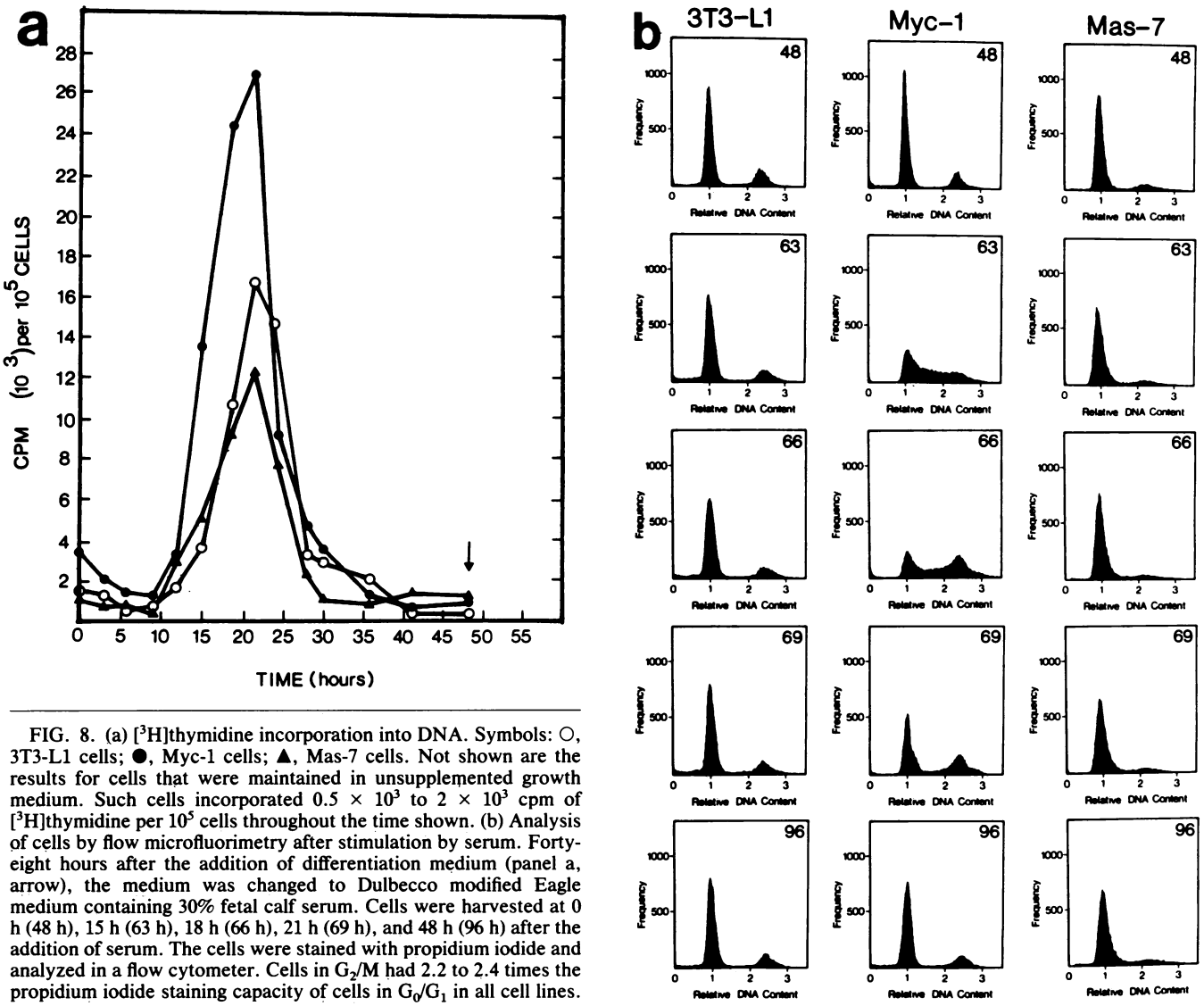

FIG. 8. (a) [^{3}H]thymidine incorporation into DNA. Symbols: ○, 3T3-L1 cells; ●, Myc-1 cells; ▲, Mas-7 cells. Not shown are the results for cells that were maintained in unsupplemented growth medium. Such cells incorporated 0.5×10^3 to 2×10^3 cpm of [^{3}H]thymidine per 10^5 cells throughout the time shown. (b) Analysis of cells by flow microfluorimetry after stimulation by serum. Forty-eight hours after the addition of differentiation medium (panel a, arrow), the medium was changed to Dulbecco modified Eagle medium containing 30% fetal calf serum. Cells were harvested at 0 h (48 h), 15 h (63 h), 18 h (66 h), 21 h (69 h), and 48 h (96 h) after the addition of serum. The cells were stained with propidium iodide and analyzed in a flow cytometer. Cells in G_2/M had 2.2 to 2.4 times the propidium iodide staining capacity of cells in G_0/G_1 in all cell lines.

are necessary for cells to become competent to replicate DNA. These events could represent induction (repression) of other genes that participate in the competence phase of the cell cycle (31, 32). All cell lines traversed the cell cycle with similar kinetics upon initiation of the differentiation program and subsequently arrested in G_0/G_1. Therefore, among these cell lines, no significant difference was detected in their ability to arrest growth in G_0/G_1 or to replicate their genomes in the presence of inducing agents.

These findings led to the hypothesis that the *myc*-induced block is manifested after cells traverse the cell cycle in the presence of inducing agents. Confluent monolayers of 3T3-L1 cells and Mas cells lost their responsiveness to high concentrations of serum after a 48-h treatment with differentiation inducers. It is likely that such cells were initiating the commitment process in which they irreversibly withdraw from the cell cycle and terminally differentiate without further exposure to inducers. In contrast, cells expressing high constitutive levels of *myc* RNA reentered S phase when exposed to 30% serum after treatment with differentiation inducers. Such cells were not capable of withdrawing from the cell cycle and thus failed to terminally differentiate. These findings indicate that normal 3T3-L1 cells enter a distinct state in G_0/G_1 after treatment with differentiation inducers into which cells expressing high levels of *myc* RNA are precluded from entering. Although the exact nature of this state is unknown, it probably reflects a unique composition of gene expression rather than a distinct temporal position in G_0/G_1. It was previously shown that arrested monolayers of BALB/c 3T3 T mesenchymal preadipocytes, which are very similar to 3T3-L1 cells, do not traverse the cell cycle when challenged with 30% serum after treatment with agents that promote differentiation (45, 53). It was proposed that after exposure to differentiation inducers, cells arrest in G_0/G_1 in a unique state designated G_D. The G_D state is distinct from that of cells arrested at confluence or deprived of serum (designated G_S), and entry into the G_D state is a requisite for 3T3 T mesenchymal cell differentiation. The results presented in this study are consistent with the proposal that mouse preadipocytes can arrest in G_0/G_1 in two distinct states depending on whether they are exposed to agents that promote differentiation. In addition, this study extends previous findings by showing that enforced expression of c-*myc* precludes entry into the proposed G_D state.

A previous study with rat skeletal myoblasts showed that irreversible suppression of c-*myc* gene expression is not a requirement for withdrawal from the cell cycle, a condition that is a requisite for terminal differentiation (10). The c-*myc* gene is inducible by serum in both biochemically and terminally differentiated myotubes, although the degree of induction is significantly reduced in differentiated myotubes compared with that in undifferentiated myoblasts. Furthermore, transient induction of c-*myc* did not suppress the differentiated phenotype. It was concluded in that study that there is no evidence for a direct role for c-*myc*, either positive or negative, in the induction or maintenance of the irreversible withdrawal of myoblasts from the cell cycle. However, it is important to note that that study does not exclude the possibility that a transient reduction of c-*myc* mRNA levels, at a specific stage in the differentiation program, is necessary for irreversible withdrawal from the cell cycle. The results presented in the present study are consistent with such a proposal. In contrast to normal cells, cells expressing high constitutive levels of *myc* RNA did not withdraw from the cell cycle and failed to differentiate. Such cells were responsive to the mitogenic effects of serum at a stage in the differentiation program at which normal cells were not. I speculate that a transient reduction of c-*myc* gene expression, at a specific stage in the differentiation program, is necessary for initiation of the commitment process and that enforced expression of c-*myc* precludes this event. When Myc-1 cells are treated briefly with cycloheximide after exposure to inducing agents they still fail to differentiate (unpublished observations). This is in sharp contrast to results with mouse erythroleukemic cells expressing high levels of *myc* RNA. In that study, brief treatment with cycloheximide after exposure to inducing agents alleviated the block to the terminally differentiated state (6). Presumably, the affect of cycloheximide was due to a reduction in *myc* protein levels, although this was not examined. Based on this experiment it was concluded that constitutive expression of c-*myc* blocks mouse erythroleukemic cell differentiation but not commitment. Although the time and length of exposure to cycloheximide may be critical and experiments using metabolic inhibitors must be interpreted with caution, the finding that cycloheximide does not alleviate the block to differentiation in Myc-1 cells supports the proposal that enforced expression of c-*myc* blocks 3T3-L1 cell commitment.

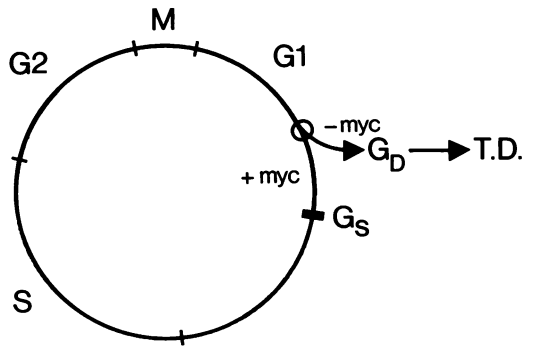

FIG. 9. Diagram of the cell cycle and *myc* control point. G_D, A state in G_0/G_1 in which cells have initiated the commitment process and will terminally differentiate without further exposure to differentiation inducers; G_S, the point in G_0/G_1 at which cells arrest when deprived of serum or when density arrested at confluence; T.D., the terminally differentiated state. +myc indicates the path taken by cells expressing high levels of *myc* RNA; −myc indicates a possible path taken by cells expressing low levels of *myc*, RNA.

***myc* is a molecular switch.** Based on results presented in this study and in others, I propose that c-*myc* is a molecular switch directing cells either to a pathway that can lead to continued proliferation or to terminal differentiation (Fig. 9). High expression of c-*myc* precludes cells from entering a predifferentiation state (G_D) and compels them to cycle. It is unclear whether the *myc*-induced block at the molecular level occurs before or after cells are exposed to inducing agents. 3T3-L1 cells expressing high constitutive levels of c-*myc* RNA are different than normal cells even before treatment with inducers of differentiation. This statement is based on our findings that Myc-1 cells have reduced levels of pro-α 2(I) collagen mRNA compared with those in normal cells (Freytag and Pogulis, submitted). This repression occurs at the transcriptional level, indicating that the genetic program of these cells has been altered. However, the present study clearly shows that the effect of enforced expression of c-*myc* on terminal differentiation is manifested after cells are exposed to inducers of differentiation. Thus, high constitutive levels of c-*myc* RNA may preclude withdrawal from the cell cycle, a condition that is a requisite for terminal differentiation. This proposal is entirely consistent with the findings that enforced expression of c-*myc* in B lymphocytes produces an overrepresentation of premature B cells in bone marrow (30).

What events are necessary for 3T3-L1 cell differentiation? This study defines the events that are necessary and sufficient for 3T3-L1 cell differentiation. The results indicate that cessation of proliferation and DNA replication in the presence of inducing agents are not sufficient for terminal differentiation in this system. In addition, cells must arrest in G_0/G_1 in a state which is distinct from that of untreated cells. What are the differences between these two states? Brown (4) has proposed that replication of the genome in the presence of inducing agents produces stable structural changes in chromatin that allow for expression of the new genetic program. Although this hypothesis is very appealing, DNA replication is not necessary for terminal differentiation in many cell types, including BALB/c 3T3 T mesenchymal preadipocytes (33, 40, 45). Therefore, DNA replication does not appear to be a universal requirement for terminal differentiation, and it is uncertain whether changes in chromatin structure, induced by replication of the genome, are important distinguishing features of these two states. Alternatively, one important difference could be the potential to resume proliferation in response to growth factors. Uncommitted cells (e.g., density-arrested cells at confluence) are ambivalent toward the proliferative or terminally differentiated state. Such cells have the option to resume proliferation or to withdraw from the cell cycle and progress to the terminally differentiated state. The path taken depends upon signals received from the environment. In contrast, committed cells withdraw from the cell cycle and can pursue only one pathway, that leading to the terminally differentiated state. Preliminary experiments have indicated that the c-*myc* gene is refractory to induction by 30% serum after 3T3-L1 cells traverse the cell cycle in the presence of inducing agents (unpublished observations). Thus, it is possible that an important step in terminal differentiation is the uncoupling of extracellular signals from events that occur in the nucleus. This could be achieved in many ways, such as the loss of growth factor receptors on the plasma membrane or prevention of the transduction of extracellular signals to the nucleus. Whatever the mechanism, it is certain that a mo-

lecular analysis of these two states will provide insight into the mechanism of commitment and terminal differentiation.

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