Regulation of c-myc Transcription and mRNA Abundance by Serum Growth Factors and Cell Contact*

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We describe effects of serum insufficiency and cell contact on the transcription and abundance of the c-myc proto-oncogene mRNA in BALB/c 3T3 fibroblasts. In exponentially growing cells, withdrawal of serum caused a 10-fold decline in c-myc mRNA within 90 min. At least part of this decline was due to a decrease in the level of myc gene transcription. These cells became quiescent at subconfluence after 36–40 h. Cells made quiescent at subconfluence or confluence contained low levels of c-myc mRNA which rose more than 20-fold 2 h after stimulation of growth by fresh serum. Thereafter, the mRNA level declined. In subconfluent cells, it declined to the level in exponentially growing cells, i.e., nearly 10-fold over the level in quiescent cells. In confluent cells, by contrast, the mRNA returned to near-quiescent levels within 18 h (by mid-S phase). However, c-myc gene transcription was regulated identically in subconfluent and confluent cultures; quiescent cells transcribed c-myc at detectable levels, and stimulation by serum caused a 5-fold increase in 1 h, followed by a decline to about 2-fold over the quiescent level within 18 h. Thus, confluence affected steady state mRNA levels without affecting the level of transcription. Our results suggest that extracellular conditions that modulate cell proliferation (serum and cell contact) exert strong and rapid control over c-myc mRNA by post-transcriptional and transcriptional mechanisms.

Cell proliferation is a highly regulated process that depends largely upon the appropriate extracellular signals. Two such signals, cell-to-cell contacts and the hormones and growth factors found in serum, have long been recognized as important modulators of normal cell growth both in vivo and in culture (1–4). The proliferation of many types of anchorage-dependent cells is suppressed by high cell density or by a suboptimal concentration of growth factors. By contrast, tumor cells, which show a loss or relaxation of growth control, often fail to arrest growth under these same conditions (4, 5).

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The mechanisms by which these environmental factors act are a subject of intense interest.

In recent years, it has become apparent that a small class of genes, collectively known as proto-oncogenes, may play a pivotal role in the control of cell proliferation (6). One such gene is c-myc, which encodes an unstable protein localized in the cell nucleus (7, 8). Transfection (9) and microinjection (10) experiments suggest that this gene is an important, but not the sole, regulator of normal (non-tumorigenic) cell proliferation. Moreover, unregulated myc expression can contribute to the tumorigenic phenotype (11, 12).

In several cell systems, the level of c-myc expression depends upon the cellular growth state. For example, the c-myc mRNA level is very low in quiescent 3T3 cells, human lymphocytes, and adult rat liver; it rises manyfold within a few hours after mitogenic stimulation of the cultured cells or partial hepatectomy (13–15). In addition, where c-myc has been implicated in the establishment or maintenance of a variety of malignant tumors, the most common myc-associated abnormality is a loss of regulation, rather than a structural mutation (6, 16).

The evidence to date suggests that c-myc may be an important growth regulator whose expression in normal cells must, in turn, be regulated by extracellular signals that modulate proliferation. To test this notion, we examined the effects of cell contacts and serum insufficiency on c-myc transcription and mRNA levels. We used for our studies non-tumorigenic BALB/c 3T3 fibroblasts (A31 cells), a cell line whose growth is very sensitive to the culture density and the serum concentration. Our results demonstrate acute negative control over c-myc expression by both cell contact and serum insufficiency.

EXPERIMENTAL PROCEDURES

Cell Culture—BALB/c 3T3 fibroblasts (A31 cells) were grown in Dulbecco's modified Eagle's medium (DME medium1) supplemented with 4 mM glutamine and 10% calf serum at 37 °C in a water-saturated 10% CO2 atmosphere. We have recently confirmed that our subclone does not form tumors in syngeneic or nude mice.

Autoradiography of Nuclei—Cells were labeled with [3H]thymidine (2 £Ci/ml; 80 Ci/mmol) and processed for autoradiography of labeled nuclei as previously described (17).

RNA Isolation—Total (nuclear and cytoplasmic) RNA was isolated by lysing cells in guanidine HCl according to the method of Cox (18) and Strohman et al. (19). Cytoplasmic RNA was isolated by lysing cells in non-ionic detergent according to the method of Pavaloro et al. (20). After extraction with phenol-chloroform (1:1), the RNA was precipitated in 70% ethanol and 200 mM NaCl, collected by centrifugation, lyophilized, and dissolved in water at 1–5 mg/ml. RNA was quantitated by absorbance at 260 nm, and purity was assessed by absorbance at 290 and 310 nm. Quantitation was confirmed by fractionation on agarose-formaldehyde gels and staining the ribo-

1 The abbreviation used is: DME medium, Dulbecco's modified Eagle's medium.
Northern Analysis—15 μg of RNA were fractionated on 1% agarose gels containing 6% formaldehyde, transferred to cellulose nitrate paper, and analyzed by Northern blot hybridization as previously described (21). Quantitation was by densitometry after autoradiography onto preflashed film.

31 Nuclease Analysis—S1 nuclease quantitation of c-myc mRNA was performed by the method of Berk and Sharp (22) as modified by Favalaro et al. (20), as previously described (23). The probe was either the 8-kilobase BssHII fragment or the 0.45-kilobase BssHII-Sac1 fragment of a murine genomic c-myc BamHI fragment cloned into pBR322 (22). The probe contained part of exon 2 and 3' adjacent before message levels declined in serum-free medium. This probe was hybridized to plasmid DNA immobilized on cellulose nitrate paper, and analyzed by Northern blot hybridization as previously described (21). Quantitation was by densitometry after autoradiography onto preflashed film, and quantitated by densitometry.

Transcription Assays—Nuclei were isolated and analyzed for run-on transcripts by modification of the methods of Greenberg and Ziff (25), as previously described (23). Briefly, nuclei were incubated with [32P]UTP for 30 min, which was within the linear range of 32P incorporation into RNA over time. Labeled RNA was isolated and hybridized to plasmid DNA immobilized on cellulose nitrate paper. α-Amanitin (2 μg/ml) reduced radiolabel incorporation by 20% and totally abolished sequence-specific hybridization.

DNA Probes—The probe used for S1 analyses was the 5.5-kb BamHI fragment of a murine genomic clone containing exons 2 and 3, provided by Dr. P. Leder (24); for transcription assays, a murine cDNA provided by Dr. K. Marcu (26) was used. The c-myc probe was a 1-kb rat cDNA given by Dr. S. Farmer (27), the histone probe was either the histone H3.2 or histone 2B cDNA used with comparable results and given by Dr. W. Marzluff (28), and the JE probe was a cDNA clone provided by Dr. C. Stiles (29).

Materials—Culture dishes were from Falcon or Lux, media was from Flow Laboratories, and serum was from Hyclone Laboratories. Radioisotopes were from New England Nuclear or ICN. Restriction enzymes were from New England Biolabs and S1 nuclease was from Boehringer Mannheim. Cycloheximide and actinomycin D were from Sigma.

RESULTS

Response to Serum Removal—Proliferating A31 cells express c-myc mRNA at easily detectable levels (11, 30), while quiescent A31 cells express this mRNA at very low levels (11, 12, 30). In the absence of adequate serum, A31 cells become quiescent within 36–40 h (31). Therefore, we asked how quickly and at what level does c-myc expression respond to serum insufficiency in proliferating A31 cells?

Cytoplasmic RNA was isolated from exponentially growing A31 cells and at various times after the cells had been washed and incubated in serum-free medium containing no growth factors. The relative abundance of c-myc mRNA was determined by S1 nuclease analysis, and the relative abundance of actin mRNA was determined by Northern blot analysis (Fig. 1A). After 45 min in serum-free medium, the c-myc mRNA level had declined about 3-fold. This was close to the decline seen when these cells were incubated for 45 min with 10% serum and actinomycin D (5 μg/ml), a strong transcriptional inhibitor (>95% inhibition of [3H]uridine incorporation). By contrast, the actin mRNA level was either unaltered during treatment with serum-free medium or, in some experiments, it increased about 2-fold; actinomycin D had no effect on actin mRNA or cell viability during the course of the experiment.

The decay kinetics of c-myc mRNA in the presence of actinomycin D or in serum-free medium are shown by Fig. 1B. The half-life of the message was approximately 20 min in the presence of actinomycin D and approximately 25 min in serum-free medium. A short lag (about 10 min) was seen before message levels declined in serum-free medium. This may reflect the time needed to clear a significant fraction of the occupied growth factor receptors from the cell surface.

The level of c-myc mRNA in isolated nuclei also declined rapidly after serum was removed from proliferating cells. Nuclei were isolated from control cells and after 45 min of incubation in serum-free medium. 32P-Labeled nuclear run-off transcripts were analyzed for c-myc, JE (a platelet-derived growth factor-inducible gene (29)), actin, and histone sequences to estimate the level of transcription at the time of nuclear isolation (Fig. 2). Within 45 min of incubation in serum-free medium, c-myc transcription declined about 2-fold to about the level observed in quiescent cells (see below). By
contrast, the transcription of histone and JE was unaltered. Surprisingly, actin transcription also showed a rapid decline (4-5-fold). Thus, c-myc mRNA abundance and transcription and actin transcription decreased very rapidly in the absence of serum.

Growth-dependent Regulation in Quiescent Subconfluent Cultures—Proliferating A31 cells were made quiescent at subconfluence (initially about 25% confluent) by serum deprivation (0.4% serum for 48 h). Under these conditions, the culture became completely quiescent (17, 31), and the cells occupied about 40% of the culture dish surface area. These cells were then stimulated to proliferate by addition of fresh medium containing 15% serum. We have shown that the first cells initiate DNA synthesis about 12 h after stimulation, most of the cells are in mid-S phase about 18 h after stimulation, and a second less synchronous wave of DNA synthesis occurred about 28-30 h (32).

Total cellular RNA was isolated at various times after stimulation and analyzed for c-myc, actin, and histone mRNA; parallel cultures were pulsed with [3H]thymidine to monitor progress through S phase (Fig. 3). As expected (13, 14, 30), c-myc mRNA abundance was very low in quiescent cells, and it rose 20-30-fold within 2 h of stimulation. Over the next 6-8 h, the message steadily declined from its peak to between 7- and 10-fold over the quiescent level. This level was maintained without fluctuation as the cells completed S, G2, and mitosis and entered G1 of the next cell cycle. Therefore, c-myc mRNA fluctuated with growth state but not with position in the cell cycle once cells were activated to proliferate (30, 33, 35).

Actin mRNA was present in fairly high abundance in quiescent cells, and it increased 2-3-fold shortly after growth stimulation, as previously reported (13, 34). Thereafter, the message levels did not appreciably fluctuate. By contrast, histone mRNA was not detected in quiescent cells but was present at substantial levels as the population progressed through S phase (28).

We next measured the transcription of c-myc, JE, actin, and histone in nuclei isolated from quiescent and stimulated subconfluent cells (Fig. 4A). c-myc transcription rose 5-fold (occasionally as high as 10-fold) within 1 h of stimulation, in agreement with the findings of Greenberg and Ziff (25). However, by 18 h after stimulation, transcription had declined to about 2-fold over the quiescent level, and this was about the level observed in proliferating cells. A similar rise and fall in transcription was shown by actin and JE. Histone transcription, by contrast, was fairly high in quiescent cells and reproducibly decreased 1 h after stimulation; by 18 h and during exponential growth, transcription was only slightly above the quiescent level. Others have noted detectable levels of histone transcription in cells in Go (25) or G1 (28, 36) and have shown that post-transcriptional mechanisms strongly regulate histone mRNA levels (28, 36, 37).

In summary, the above results show that when subconfluent cells were stimulated from quiescence there was a transient increase in c-myc mRNA and transcription that exceeded the level maintained by proliferating cells. After this burst, transcription was maintained at about 2-fold over the level in the quiescent cells, while cytoplasmic mRNA was maintained at 7–10-fold over the quiescent level.

Regulation in Confluent Cultures—We next followed c-myc mRNA levels in cultures made quiescent by growth to confluence. Fig. 5 shows the cytoplasmic c-myc, actin, and histone mRNA abundance in cultures made quiescent by one of three means: serum deprivation at subconfluence (as described above), growth to confluence in 10% serum, or serum deprivation after growth to confluence. These cultures were then stimulated with fresh medium containing 15% serum, and

![Fig. 3. c-myc, actin, and histone mRNA in synchronized subconfluent cells.](image-url)

![Fig. 4. c-myc, JE, actin, and histone transcription in quiescent, stimulated, and proliferating cells.](image-url)
cytoplasmic RNA was isolated 2 and 18 h later. All three cultures contained very low levels of c-myc mRNA prior to stimulation; 2 h after stimulation, c-myc mRNA showed a substantial rise, actin mRNA showed a 2-3-fold induction, and histone mRNA was not detected. However, 18 h after stimulation, only the subconfluent culture expressed c-myc mRNA substantially (about 10-fold) above the quiescent level. Confluent cells, whether or not they had been serum-deprived, showed a marked reduction in this mRNA, such that the level at 18 h was close to that found in quiescent cells. Prior serum deprivation of confluent cells always enhanced the mitogenic response, evidenced by the per cent of labeled nuclei and level of histone mRNA present at 18 h. The most important difference between subconfluent and confluent cultures is that while the former undergo more than one population doubling after stimulation, the latter undergo less than one population doubling. In confluent cultures, regardless of the availability of serum growth factors, c-myc mRNA is "superinducible" by cycloheximide, an inhibitor of protein synthesis (14, 16). In another cell system, a post-transcriptional site of "superinduction" by protein synthesis inhibitors was demonstrated (38).

To determine the effects of cycloheximide in A31 cells, cytoplasmic RNA was isolated from proliferating cells and 1 h after treatment with either cycloheximide (2 μg/ml; 90% inhibition of protein synthesis), actinomycin D (5 μg/ml); or both drugs (Fig. 6A). Cycloheximide increased c-myc mRNA about 3-fold, while actinomycin D caused a substantial decrease in the message level, as expected. However, in the presence of both drugs the level of mRNA remained constant, that is cycloheximide stabilized the cytoplasmic message against the decay usually observed in the presence of actinomycin D.

**Effect of Cycloheximide**—One final set of experiments lends support to the idea that c-myc is subject to strong post-transcriptional regulation in A31 cells. It has been shown that c-myc mRNA is "superinducible" by cycloheximide, an inhibitor of protein synthesis (14, 16). In another cell system, a post-transcriptional site of "superinduction" by protein synthesis inhibitors was demonstrated (38).

![Fig. 6. Effect of cycloheximide (CHM) on c-myc mRNA and transcription.](attachment:fig6.png)

A. C-MYC mRNA

![Proliferating Cells](attachment:proliferating_cells.png)

B. C-MYC mRNA

![Quiescent Cells](attachment:quiescent_cells.png)

C. Transcription

![Histone](attachment:histone.png)
molecules occupying a particular gene, which clearly determines in part the level of transcription. However, additional factors, including those regulating the processing and transport out of the nucleus in the intact cell, are not measured by the run-off assay, nor is the in situ rate of transcription measured by this assay. It is difficult, therefore, to determine the relative contributions made by transcriptional and post-transcriptional regulation to the more than 20-fold rise in the cytoplasmic c-myc mRNA level that occurs shortly after serum stimulation. However, taken together, our data are consistent with a role for both transcriptional and post-transcriptional regulation of c-myc mRNA levels in A31 cells.

During asynchronous growth, serum was required for maintenance of both the mRNA and transcriptional level of the c-myc gene. When serum is removed from proliferating A31 cells, progress through the cell cycle continues unperturbed for 2-3 h; thereafter, cells no longer initiate DNA synthesis (enter S phase), although cells in S or G2 complete the remainder of the cycle through mitosis (31). It is, therefore, of interest that c-myc mRNA responded so quickly to serum withdrawal. This rapid response was not seen with actin mRNA, and it is not observed with the ornithine decarboxylase mRNA. Since the c-myc protein is also very labile (7), repression of gene expression may be an early acute response to serum deprivation that triggers the ultimate cessation of growth. It was also surprising that actin transcription, but not actin mRNA levels, rapidly declined after serum withdrawal. We have noted that serum withdrawal induces a transient morphological change (cell rounding), and it remains a possibility that cell rounding plays an important role in the selective decline in c-myc and actin transcription and c-myc mRNA.

Recently, an exclusive role for post-transcriptional regulation of c-myc mRNA has been demonstrated in two cell systems: thrombin-stimulated hamster fibroblasts (39) and terminally differentiated F9 teratocarcinoma stem cells (23, 40). In addition, c-myc mRNA levels were shown to be unresponsive to growth state in cultured avian fibroblasts (33). It is not clear at this time whether these results reflect differences in cell type or whether they reflect differences in the agents used to induce growth arrest or growth stimulation. Nonetheless, in A31 cells, the c-myc mRNA level fluctuated with growth state, and the cellular environment markedly influenced the level of this mRNA. We suggest that environmental factors that regulate cell proliferation may do so by regulating in an acute manner specific genes such as c-myc.

REFERENCES

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Growth Regulation and c-myc Expression