Loss of Protooncogene c-Myc Function Impedes G₁ Phase Progression Both before and after the Restriction Point

Christoph Schorl and John M. Sedivy*

Department of Molecular Biology, Cell Biology, and Biochemistry, Brown University, Providence, Rhode Island 02912

Submitted October 11, 2002; Revised October 11, 2002; Accepted November 6, 2002
Monitoring Editor: Mark J. Solomon

c-myc is an important protooncogene whose misregulation is believed to causally affect the development of numerous human cancers. *c-myc* null rat fibroblasts are viable but display a severe (two- to threefold) retardation of proliferation. The rates of RNA and protein synthesis are reduced by approximately the same factor, whereas cell size remains unaffected. We have performed a detailed kinetic cell cycle analysis of *c-myc*^{-/-} cells by using several labeling and synchronization methods. The majority of cells (>90%) in asynchronous, exponential phase *c-myc*^{-/-} cultures cycle continuously with uniformly elongated cell cycles. Cell cycle elongation is due to a major lengthening of G₁ phase (four- to fivefold) and a more limited lengthening of G₂ phase (twofold), whereas S phase duration is largely unaffected. Progression from mitosis to the G₁ restriction point and the subsequent progression from the restriction point into S phase are both drastically delayed. These results are best explained by a model in which c-Myc directly affects cell growth (accumulation of mass) and cell proliferation (the cell cycle machinery) by independent pathways.

INTRODUCTION

The deregulation of *c-myc* gene expression is frequently observed in many diverse human malignancies (Henriksson and Luscher, 1996; Dang, 1999). In normal cells the expression of the *c-myc* protooncogene is under tight control and is rapidly induced by mitotic stimuli and suppressed by negative growth signals (Obaya *et al.*, 1999; Grandori *et al.*, 2000). The c-Myc protein is a transcription factor whose DNA-binding domain contains basic region, helix-loop-helix, and leucine zipper motifs (Luscher and Larsson, 1999). c-Myc forms an obligate heterodimer with its partner Max and binds to a DNA consensus sequence known as an E-box [CA(C/T)GTG] (Blackwood and Eisenman, 1991; Prendergast and Ziff, 1991; Amati and Land, 1994). The first 143 N-terminal amino acids comprise a regulatory domain that can both positively and negatively modulate the expression of target genes (Claassen and Hann, 1999; Amati *et al.*, 2001). The mechanisms by which c-Myc modulates target gene transcription are not well understood (Oster *et al.*, 2002); activation of target genes has been suggested to involve processes that impact histone acetylation (McMahon *et al.*,

1998; McMahon *et al.*, 2000; Frank *et al.*, 2001), ATP-dependent chromatin remodeling (Cheng *et al.*, 1999; Wood *et al.*, 2000), and promoter clearance (Eberhardy and Farnham, 2001). Repression of target genes has been reported to involve interference with the initiator element (Inr)-binding activator Miz-1 (Staller *et al.*, 2001), but Inr-independent repression has also been reported by several groups (Fachini *et al.*, 1997; Xiao *et al.*, 1998; Yang *et al.*, 2001).

c-myc knockout mice display numerous developmental abnormalities and die at day 10.5 of gestation (Davis *et al.*, 1993). A homozygous *c-myc* knockout in a rat fibroblast cell line is not lethal but the cells display a severe retardation of proliferation (Mateyak *et al.*, 1997). *Drosophila c-myc* null mutants have not been reported, but hypomorphic null alleles display a diminutive phenotype and female sterility (Gallant *et al.*, 1996; Schreiber-Agus *et al.*, 1997). A mosaic analysis in the wings showed that ablation of dMyc expression resulted in a decrease in cell size but not in the number of cells, whereas overexpression caused cells to become larger without affecting the rate of cell division (Johnston *et al.*, 1999). Further results from mammalian cells showing that c-Myc overexpression can trigger an increase in cell size (Iritani and Eisenman, 1999; Beier *et al.*, 2000) led to proposals that the primary target of Myc activity may be the accumulation of cell mass (Elend and Eilers, 1999; Schmidt, 1999; Schuhmacher *et al.*, 1999). However, more recent work utilizing a series of hypomorphic alleles as well as condi-

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E02-10-0649. Article and publication date are at www.molbiol.org/cgi/doi/10.1091/mbc.E02-10-0649.

* Corresponding author. E-mail address: john_sedivy@brown.edu.

tional deletion of *c-myc* in the mouse argues that Myc controls cell number but not cell size (Trump *et al.*, 2001). Expression profiling of *c-Myc* target genes has not adequately addressed this issue to date, because genes encoding cell cycle regulators, metabolic enzymes, as well as ribosomal proteins were found to be affected (Coller *et al.*, 2000; Greasley *et al.*, 2000; Guo *et al.*, 2000; Nesbit *et al.*, 2000; O'Hagan *et al.*, 2000).

What is critically needed are additional studies addressing the influence of *c-Myc* on various aspects of cellular physiology. A cell culture model in which both copies of the endogenous *c-myc* gene have been deleted by gene targeting in a rat fibroblast cell line (Mateyak *et al.*, 1997) has been used extensively to investigate the roles of *c-Myc* in the regulation of transcription, cell cycle progression, and apoptosis (Mateyak *et al.*, 1999; Frank *et al.*, 2001; Soucie *et al.*, 2001; Staller *et al.*, 2001). Loss of *c-myc* reduced the rate of cell division two- to threefold (Mateyak *et al.*, 1997). Interestingly, the rate of RNA and protein synthesis was reduced by approximately the same factor, whereas cell size remained largely unaffected. A preliminary analysis of cell cycle progression indicated that G_1 and G_2 phases were elongated, whereas the duration of S phase remained relatively constant (Mateyak *et al.*, 1997). However, this analysis was limited to a single method and did not measure kinetically the duration of individual cell cycle phases, nor did it address whether all cells cycled with similar kinetics. We therefore undertook a detailed cell cycle analysis of *c-myc*^{-/-} cells by using several labeling and synchronization methods. We report herein that the majority (>90%) of cells in asynchronous, exponential phase *c-myc*^{-/-} cultures cycle continuously with uniformly elongated cell cycles. Cell cycle elongation is due to a major lengthening of G_1 phase and a more limited lengthening of G_2 phase, whereas S phase duration is largely unaffected. The lengthening of G_1 is caused by, in essentially equal measure, a delay in passage through the restriction (R) point as well as the slowing of the subsequent progression into S phase.

MATERIALS AND METHODS

Cell Culture

TGR-1 (*c-myc*^{+/+}) and HO15.19 (*c-myc*^{-/-}) cells were grown in DMEM medium supplemented with 10% calf serum (CS) as described previously (Mateyak *et al.*, 1999). Unless otherwise stated, all experiments were performed using continuously cycling asynchronous cultures. Great care was taken to ensure that cultures were maintained in continuous exponential growth with frequent medium changes and at low cell density for many generations. To avoid cell-cell contact cultures were split 1:2 (HO15.19) or 1:3 to 1:5 (TGR-1) after reaching 30% confluence. This subculture regimen was maintained continuously in 10-cm dishes for up to 15–18 passages. Cells were used as needed for various experiments by seeding into appropriate culture vessels. The mitotic fraction of cells was harvested by mechanical shake-off without the use of drugs. To collect mitotic cells a large number of 10-cm dishes containing asynchronously cycling, exponential phase cultures were gently tapped against a hard surface. The medium was collected, cells were recovered by brief low-speed centrifugation, resuspended in a small amount of medium, and plated into 24-well microtiter dishes. Growth curves were performed by seeding 55,000 cells (~10% confluence) per 6-cm dish and incubation in an atmosphere of either 20 or 2% O₂. Every 24 h cells from three plates were harvested with trypsin. Cells collected from each dish were counted in duplicate by

using a Coulter Counter. To analyze the effect of cell density on cell cycle progression cells were seeded at densities ranging from 2500–25,000 cells/cm² and pulse-labeled with BrdU (5-bromo-deoxy-uridine) 16 or 48 h after plating.

BrdU Incorporation Assays

Labeling experiments were performed in 24-well microtiter plates unless indicated otherwise. For continuous BrdU labeling, cells were seeded at 3000–4000 cells/well (700–1000 cells/cm² or ~5% confluence), and 12–14 h later the culture medium was removed, cells were washed once in Dulbecco's phosphate-buffered saline (PBS) lacking magnesium and calcium, and medium containing 1 µg/ml BrdU (3.2 µM) and 1 mg/ml uridine (4.1 mM) was added. In mitotic shake-off experiments harvested cells were allowed to attach for 2 h (TGR-1) or 4 h (HO15.19) before BrdU labeling (thirty 10-cm dishes yielded sufficient mitotic cells for 12 wells of a 24-well microtiter plate). BrdU incorporation was stopped at the indicated time points by adding 0.4 M ascorbic acid (to a final concentration of 67 mM) to the culture medium (Moscovitis and Pardee, 1980). Pulse labeling with BrdU was performed as detailed above, except that the labeling was stopped after 15, 30, or 60 min as indicated in the figure legends. Cells were harvested immediately after the pulse unless otherwise indicated. To observe labeled mitoses cells were seeded in 6-cm dishes at 700–1000 cells/cm², pulse labeled with BrdU for 30 min, washed twice with PBS, and the incubation was continued in fresh medium containing 8 µg/ml (32 µM) thymidine. All BrdU-containing cultures were handled in a darkened room with only indirect orange illumination.

In Situ Histochemical Staining of BrdU Incorporation

Plates were washed three times with PBS, and the cells were fixed for 10 min with ice-cold 100% methanol. After three further PBS washes, DNA was denatured using 1.5 M HCl for 1 h at room temperature. The HCl was neutralized by three washes over a 10-min period (~3 min/wash) with 0.1 M borate buffer, pH 8.5, followed by three further PBS washes. Blocking was performed with PBS containing 0.1% (wt/vol) bovine serum albumin (PBSA) for 1 h at 37°C. Incubation with anti-BrdU monoclonal antibody (mAb) (catalog no. 555 627; BD PharMingen, San Diego, CA) was for 1 h at 37°C. Antibody was diluted 1:100 to 1:200 (5–2.5 µg/ml) in PBSA. Subsequently, the Vectastain Elite ABC and Novared kits (Vector Laboratories, Burlingame, CA) were used according to the manufacturer's instructions.

Double Staining for 5-Chloro-deoxy-uridine (CldU)- and 5-Iodo-deoxy-uridine (IdU)-positive Cells

This procedure was adapted from a protocol described by Aten *et al.* (1992). Exponentially growing cells in 24-well microtiter plates were washed twice in PBS followed by a 30-min incubation in medium containing 10 µM (2.6 µg/ml) CldU. After the labeling the cells were washed twice in PBS and then incubated for 1 h in medium containing 200 µM thymidine (48.5 µg/ml). After two brief washes with PBS incubation was continued in unsupplemented medium. At the indicated time points, the medium was removed, cells were washed twice with PBS, and incubated for 30 min in medium containing 10 µM (3.5 µg/ml) IdU, at which point the cells were fixed and denatured as described above. Neutralized cells were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked for 1 h at 37°C in PBS-T containing 1% BSA (PBSA-T). Incubation with the primary antibodies was for 1 h at room temperature. CldU was detected with the anti-BrdU rat mAb [clone BU1/75 (ICR1), catalog no. MAS 250; Harlan, Indianapolis, IN] diluted 1:520 in PBSA-T, followed by Cy-3-conjugated donkey anti-rat secondary antibody (catalog no. 712-165-153; Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:200 (3.0 µg/ml). IdU

was detected with the mouse monoclonal anti-BrdU antibody (clone B44; catalog no. 347 580; BD Biosciences, San Jose, CA; Gratzner, 1982) diluted 1:10 in PBSA-T (2.5 $\mu\text{g}/\text{ml}$), followed by Alexa 488-conjugated goat anti-mouse secondary antibody (catalog no. A-11029; Molecular Probes, Eugene, OR) diluted 1:600 (3.0 $\mu\text{g}/\text{ml}$). Incubation with each antibody was for 1 h at room temperature. After each antibody incubation cells were treated for 10 min in high salt buffer (28 mM Tris, pH 8.0, 500 mM NaCl, 0.5% Tween 20) followed by a 10-min wash in PBSA-T. Cells were counterstained with 0.1 $\mu\text{g}/\text{ml}$ 4,6-diamidino-2-phenylindole for 15 min, washed twice with PBS, and stored in PBS at 4°C.

Flow Cytometry

Flow cytometric analysis was performed on a BD Biosciences FACS-Calibur instrument by using CellQuest and Modfit software. Excitation was at 488 nm. Alexa 488 and 5-(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) emissions were recorded in the FL-1 channel and propidium iodide (PI) and sulforhodamine 101 (SR; Molecular probes) emissions in the FL-2 channel. For PI staining, cells were harvested by trypsinization, fixed with ethanol, and stained as described previously (Shichiri *et al.*, 1993). BrdU incorporation was detected using anti-BrdU mAb B44 (BD Biosciences) as recommended by the manufacturer, followed by an Alexa 488-conjugated secondary antibody. To label cells with CFSE, exponentially cycling cultures ($1-3 \times 10^7$ cells) were harvested with trypsin, resuspended in 1 ml of complete DMEM medium containing 10 μM CFSE, and incubated at 37°C for 10 min. Fourteen milliliters of ice cold complete medium was added, mixed well, and incubation was continued on ice for 5 min. Cells were recovered by centrifugation (1500 rpm, 4°C, 5 min), resuspended in complete medium, and an aliquot was immediately analyzed for CFSE incorporation. The remaining cells were diluted, seeded at <20% confluence in multiple dishes, and incubated under standard culture conditions. One dish was harvested by trypsinization every 24 h for the duration of the experiment. Flow cytometric analysis was done on live (unfixed) cells. To label cells with SR, cells were fixed with ethanol as described for PI staining (see above), resuspended in PBS, stained with 3 $\mu\text{g}/\text{ml}$ SR for 5 min., and immediately analyzed by flow cytometry.

Microscopy and Data Analysis

Microscopic observation was performed with a Diaphot inverted microscope (Nikon, Tokyo, Japan) equipped with phase and epifluorescence optics. Histochemically stained cells were counted by visual observation of random fields under 200 $\times$ magnification. Fluorescently labeled cells were photographed with a Spot-II digital camera (Diagnostics Products, Los Angeles, CA). Cells were scored in random microscopic fields. Means and SDs (n, number of counted fields) were calculated using Microsoft Excel and graphs were generated using Cricket Graph. The minimum total number of cells in each experiment is indicated in the figure legends.

Real-Time Polymerase Chain Reaction (PCR)

RNA was prepared using the RNeasy-4PCR kit (Ambion, Austin, TX) according to the manufacturer's instructions. One microgram of total RNA was transcribed into cDNA in a 50- μl reaction by using the TaqMan kit (Applied Biosystems, Foster City, CA) and the following conditions: 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C. One microliter of this reaction was used as template in the subsequent PCR reactions by using SYBR Green Master Mix and 0.8 μM (each) of forward and reverse primer for either p21 or GAPDH. PCR was performed in a Prism 7700 sequence detector (Applied Biosystems) according to the manufacturer's instructions. Each sample/primer pair reaction was run in triplicate. Threshold values were determined for each sample/primer pair and average and SD

values were calculated. p21 expression was normalized against GAPDH expression.

Determination of the Restriction Point

To determine the restriction point during the M-to-S transitions, cells were synchronized in mitosis by shake-off and plated in 24-well microtiter wells containing serum-supplemented medium (10% CS). Cells were allowed to attach for 2 h, at which point the medium in all but one well was changed to serum-supplemented medium containing BrdU/uridine. The remaining well was carefully rinsed three times with serum-free medium and then replaced with serum-free medium containing BrdU/uridine. At the indicated time points, individual wells were switched to serum-free medium containing BrdU/uridine in an identical manner. The total incubation time after shake-off was kept constant (TGR-1, 24 h; HO15.19, 46 h) at which point the wells were fixed and processed. To determine the restriction point during the G₀-to-S transition cells were grown to confluence and subsequently serum starved for 48 h in medium containing 0.25% CS. To initiate cell cycle entry, cells were trypsinized and seeded in 35-mm dishes at ~50% confluence in 10% CS-supplemented medium. Cells were allowed to attach for 2 h, switched to serum-free medium at successive time points, and labeled with BrdU as described above. The experiment was terminated at 28 and 52 h for TGR-1 and HO15.19 cells, respectively. In some experiments, cells were made quiescent by contact inhibition only. In this case, the 48-h period of incubation in medium containing 0.25% CS (above) was replaced with an equivalent incubation period in 10% CS-supplemented medium.

RESULTS

Pulse labeling of asynchronously cycling, exponential phase cells with BrdU for 15, 30, or 60 min resulted in ~65 and 30% incorporation in *c-myc*^{+/+} and *c-myc*^{-/-} cells, respectively (Figure 1A). Flow cytometric analysis of propidium iodide-stained cells gave S-phase values in the range of 46% for *c-myc*^{+/+} cells and 18% for *c-myc*^{-/-} cells (Table 1). The discrepancy between the two measurements can be explained by the poor resolution of the flow cytometric data between early S and G₁ events, and late S and G₂ events. In contrast, because even a 5-min BrdU pulse resulted in clearly detectable *in situ* staining (our unpublished data), this method of analysis accurately captures very early as well as very late S-phase cells. Combining the BrdU-estimated S-phase values with the measurement of growth rate gives calculated S-phase durations of 12.6 and 13.7 h for *c-myc*^{+/+} and *c-myc*^{-/-} cells, respectively (Table 2). Using this BrdU-derived data set, the combined durations of G₂ + M + G₁ can then be calculated as 6.1 and 29.3 h for *c-myc*^{+/+} and *c-myc*^{-/-} cells, respectively. Assuming that in the flow cytometric data G₁ and G₂/M events are inflated equivalently with S events, increasing S- to the BrdU-derived values would shorten G₁ and G₂/M durations to 5.1 and 1.1 h for *c-myc*^{+/+} cells, and to 22.4 and 6.9 h for *c-myc*^{-/-} cells (Table 2).

Continuous labeling of asynchronously cycling cells resulted in linear rates of accumulation with plateaus of 97 and 90% reached in 10 and 24 h by *c-myc*^{+/+} and ^{-/-} cells, respectively (Figure 1B). Because the last cells to be labeled correspond to cells that have just exited S phase at the time that label was added (0 h), the time to reach the plateau is a measure of the combined lengths of G₂/M + G₁. If the line of accumulation for *c-myc*^{-/-} cells is extrapolated to 100% a value of 27.2 h is obtained, which is somewhat faster than

Figure 1. Single BrdU labeling (A and B) and double CldU/IdU labeling (C) of asynchronously cycling cells. (A) Pulse labeling with BrdU. Cells were pulse labeled for the indicated times, harvested immediately afterward, and processed as described in MATERIALS AND METHODS. (B) Continuous labeling with BrdU. BrdU was added at $t = 0$ and cells were harvested at the indicated time points. The labeling indices (means $\pm$ SD, $n = 11$ fields/time point) denote BrdU-positive cells as percentage of total cells. A minimum of 687 *c-myc*^{+/+} and 676 *c-myc*^{-/-} cells/time point (A) and 645 *c-myc*^{+/+} and 634 *c-myc*^{-/-} cells/time point (B) were scored. (C) Double labeling with CldU and IdU. Cells were pulse labeled with CldU for 30 min at $t = 0$, and culture was continued in the absence of CldU as described in MATERIALS AND METHODS. At the indicated time points, the second pulse was delivered with IdU for 30 min and the cells were harvested immediately afterward. The labeling indices (means $\pm$ SD, $n = 3\text{--}4$ [*c-myc*^{+/+}] and 6–10 [*c-myc*^{-/-}] fields/time point) denote CldU/IdU double-labeled cells as percentage of total CldU-positive cells. A minimum of 57 *c-myc*^{+/+} and 44 *c-myc*^{-/-} CldU-positive cells/time point were scored. The BrdU labeling indices in this figure were obtained using *in situ* immunohistochemistry methods.

the value of 29.3 h calculated from pulse-labeling values (Table 2). More importantly, however, the fact that BrdU-positive cells accumulate to an equivalently high plateau in both *c-myc*^{+/+} and *c-myc*^{-/-} cultures indicates that the majority of cells (>90%) are cycling in both cases.

To kinetically assess the duration of G₁ phase, synchronization in M phase was performed by mitotic shake-off. To minimize physiological perturbations cells were recovered by mechanical agitation from asynchronously cycling, exponential phase cultures without the use of drugs. Continuous labeling resulted in smooth monophasic accumulation profiles and reached a plateau of ~90% for both *c-myc*^{+/+} and *c-myc*^{-/-} cells (Figure 2A). The very low initial incorporation values (2–3%) indicate that the shake-off was minimally contaminated with interphase cells. Fastest G₁ transit times were 2 h for *c-myc*^{+/+} cells and 12 h for *c-myc*^{-/-} cells, but these events were very few, representing only 3–4% of total cells in both cases. The midpoints of the transitions fell at 4.9 and 24.7 h for *c-myc*^{+/+} and *c-myc*^{-/-} cells, respectively. These values are in very good agreement with the G₁ times of 5.2- and 22.4-h calculated on the basis of pulse labeling of asynchronous cultures (Table 2). Given that the synchrony of the *c-myc*^{-/-} culture seemed to be lower than that of the *c-myc*^{+/+} culture, the G₁ transit time of 24.7 h for *c-myc*^{-/-}

cells is likely to be an overestimate. The lower synchrony of *c-myc*^{-/-} cultures may be due to the fact that these cells took somewhat longer to attach (our unpublished data). Nevertheless, the high final incorporation values indicate that the majority of cells in both cultures were actively cycling. Furthermore, the smooth profiles of the curves indicate that no significant cohorts of differentially cycling cells were present in either culture.

Additional information on S phase as well as overall cell cycle durations can be obtained by pulse labeling of mitotic shake-off cultures. If the pulses are short, BrdU incorporation increases early as cells enter S phase and decreases at later times as cells exit into G₂. In practice, this experiment is affected strongly by the synchrony of the cultures (the higher the peak the higher the synchrony). This is because the fastest cells are no longer labeled by later pulses, bringing down the labeling index, whereas other cells are still in S phase. Thus, the time at which labeling begins to decline corresponds roughly to the duration of G₁ + S for the fastest cells. In cultures with good synchrony the labeling index increases again at later times as cells begin to enter into a second cell cycle. Fastest G₁ transit times were 2 h for *c-myc*^{+/+} cells and 10 h for *c-myc*^{-/-} cells, confirming previous results (Figure 2B). In *c-myc*^{+/+} cultures labeling first began to fall off at 12 h; given a 2–3 h G₁ phase for the fast cells this results in a 9–10 h S phase, somewhat shorter than the 12.6 h calculated for S from exponentially cycling cells (Table 2). In *c-myc*^{-/-} cultures labeling fell off at 24 h; combined with a 10- to 12-h G₁ phase for the fast cells in this experiment this gives a 12- to 14-h S phase, in good agreement with the 13.7 h calculated previously. A late increase in the labeling index occurred clearly for *c-myc*^{+/+} cells at 20 h, and this value thus corresponds to the length of G₁ + S + G₂ + M + G₁ for the fastest cells. Again, this is in good agreement with previous data. For *c-myc*^{-/-} cells, a small upswing in labeling was seen at 44 h and beyond. Although this value is also in good agreement with the reduced pro-

Table 1. Durations of cell cycle phases calculated from flow cytometric data

	Cell cycle distribution (% of total cells)		Calculated phase duration (h)	
	<i>c-myc</i> ^{+/+}	<i>c-myc</i> ^{-/-}	<i>c-myc</i> ^{+/+}	<i>c-myc</i> ^{-/-}
G1	37	59	7.0	25.4
S	47	18	8.8	7.7
G2/M	16	23	3.0	9.9

Table 2. Durations of cell cycle phases based on BrdU-labeling studies

	<i>c-myc</i> ^{+/+}			<i>c-myc</i> ^{-/-}		
	G1	S	G2/M	G1	S	G2/M
Cell cycle distributions (%) corrected for S phase using BrdU pulse labeling ^a	27	67	6	52	32	16
Cell cycle phases (h) calculated from cell cycle distributions and growth rates ^b	5.1	12.6	1.1	22.4	13.7	6.9
G ₁ phases (h) determined by the method of mitotic shake-off ^c	4.9			24.7		
S and G ₂ phases (h) determined by the method of labeled mitoses ^d		9.8	3.2		13.9	7.5

^a Cell cycle distributions shown for experiment A in Table 1 were corrected using BrdU incorporation values of 67% (*c-myc*^{+/+} cells) and 32% (*c-myc*^{-/-} cells). These values were obtained from the 15-min pulses shown in Figure 1A.

^b Corrected cell cycle distributions were combined with growth rate values (doubling times) of 18.8 h (*c-myc*^{+/+} cells) and 43.0 h (*c-myc*^{-/-} cells).

^c Data from Figure 2A.

^d Data from Figure 2C.

liferation of these cells measured by other means, the data are compromised by the deterioration of synchrony in these cultures.

Another method by which valuable kinetic data can be obtained, especially on G₂- and S-phase duration, is a procedure referred to as labeled mitoses. In this experiment asynchronously cycling, exponential phase cells are labeled with a single short pulse of BrdU, and at successive time points mitotic figures are scored for the presence or absence of the BrdU label. This procedure has the advantage that it does not use any synchronization steps, which could perturb normal cell cycle progression. The disadvantage is that mitotic figures are few in number, especially in slowly proliferating

cultures, making the experiments very time consuming to score.

A rapid rise in BrdU-positive mitoses was seen in both *c-myc*^{+/+} and ^{-/-} cultures (Figure 2C), reaching a peak of ~90 and 75%, respectively. The midpoint of the transition was at 3.2 h for *c-myc*^{+/+} cells and 7.5 h for *c-myc*^{-/-} cells. These kinetic measurements of G₂ phase duration are somewhat longer than the values of 1.1 and 6.9 h for *c-myc*^{+/+} and ^{-/-} cells, respectively, calculated previously on the basis of pulse-labeling of asynchronous cultures (Table 2). S-phase duration can be determined by the width of the peak of BrdU-positive mitoses, and this estimate depends critically on the synchrony of the cultures. The synchrony

Figure 2. BrdU labeling of cells synchronized in mitosis (A and B) and detection of labeled mitoses (C). (A) Continuous labeling. Cells were synchronized by mitotic shake-off as described in MATERIALS AND METHODS. BrdU was added at $t = 2$ h (*c-myc*^{+/+}) or $t = 4$ h (*c-myc*^{-/-}) and cells were harvested at the indicated time points. (B) Pulse labeling. Cells were pulse labeled for 30 min at the indicated times and harvested immediately afterward. The labeling indices (means $\pm$ SD, $n = 11$ fields/time point [A] and $n = 6$ fields/time point [B]) denote BrdU-positive cells as percentage of total cells. A minimum of 711 *c-myc*^{+/+} and 637 *c-myc*^{-/-} cells/time point (A) and 337 *c-myc*^{+/+} and 372 *c-myc*^{-/-} cells/time point (B) were scored. (C) Detection of labeled mitoses. Asynchronously growing cells were pulse labeled with BrdU for 30 min at $t = 0$. Culture was continued in the absence of BrdU as described in MATERIALS AND METHODS, and cells were harvested at the indicated time points. The labeling indices (means $\pm$ SD, $n = 4$ –7 [*c-myc*^{+/+}] and 3–9 [*c-myc*^{-/-}] fields/time point) denote BrdU-positive mitotic figures as percentage of total mitotic figures. A minimum of 44 *c-myc*^{+/+} and 33 *c-myc*^{-/-} mitotic figures/time point were scored. The BrdU-labeling indices in this figure were obtained using in situ immunohistochemistry methods.

with which the pulse-labeled cells proceed through the cycle is indicated by the steepness of the rise and fall of the peak. By this measure the progression of *c-myc*^{+/+} and *-/-* cultures were very similar, because even at later times the down slopes of the peaks are steep and almost parallel. At the midpoint of the rise the width of the peak (S-phase duration) was 9.8 h for *c-myc*^{+/+} cells and 13.9 h for *c-myc*^{-/-} cells. These estimates are in reasonably good agreement with the values of 12.6 and 13.7 h calculated previously (Table 2).

Although labeled mitoses can be followed for more than one cell cycle, this analysis is limited by the difficulty in finding sufficient numbers of mitotic cells. An alternative method to determine the ability of cells to enter into subsequent cell cycles is double labeling with two distinguishable thymidine analogs; this method can also provide an independent kinetic measure of overall cell cycle duration. In this experiment asynchronously cycling, exponential phase cells were labeled with a single short pulse of CldU, and at successive time points the cultures were pulse labeled with IdU (Figure 1C). Using appropriate primary antibodies, the CldU and IdU labels can be differentially visualized (MATERIALS AND METHODS). Similar to labeled mitoses, this procedure has the advantage that it does not use any synchronization steps. At early times, most cells were both CldU and IdU positive; however, as the CldU-labeled cells progressed through the cell cycle, the incidence of double-labeled cells decreased. Of particular interest is the subsequent peak of double-labeled cells, which represents the reentry into S phase of the CldU-labeled cohort of cells. This peak reached approximately equivalent values in both *c-myc*^{+/+} and *-/-* cells (72 and 62%, respectively), indicating that similar numbers of cells progressed into the second cell cycle in both cultures. One technical issue affecting this experiment is the relatively low sensitivity of IdU detection, which caused the fraction of double-labeled cells to be consistently underestimated. The peak of labeling occurred at 16 h for *c-myc*^{+/+} cells and 36 h for *c-myc*^{-/-} cells. In summary, this experiment showed that both *c-myc*^{+/+} and *-/-* cells progressed through the cell cycle uniformly and with comparable synchrony (as evidenced by the relatively steep slopes of the double-label peaks), that equivalent numbers of cells entered a second cell cycle, and that cell cycle progression was in very good agreement with proliferation rates measured by standard growth curves.

All the methods mentioned above measure length of S phase at relatively late time points in the experiment, making the data subject to uncertainties due to loss of synchrony. Indeed, all S-phase determinations presented above gave slightly longer values for *c-myc*^{-/-} cells than for *c-myc*^{+/+} cells. The rate of genome replication and thus the length of S phase is determined by both the number of active origins and the rate of elongation during DNA synthesis. Very short pulses of BrdU label replication forks and high-resolution immunofluorescence detection typically results in a distinctly punctate nuclear appearance. The number of spots, or "replication centers" is believed to be proportional to the number of active origins at that point in time (Berezney *et al.*, 2000). When exponentially cycling *c-myc*^{+/+} and *-/-* cultures were pulse-labeled with BrdU for 5 min and processed for immunofluorescence microscopic observation, no significant differences could be observed even after viewing num-

erous images (representative views are shown in Figure 3A). This result indicates that, on average, *c-myc*^{+/+} and *-/-* cells assemble equivalent numbers of replication centers during S-phase progression. To further expand this analysis, BrdU incorporation was quantitatively measured at the single cell level. Cells were pulse labeled and processed for immunofluorescence detection as described above, counterstained with propidium iodide, and analyzed by flow cytometry. In this protocol, S-phase cells are visualized as an arc of Alexa 488 (BrdU)-positive cells, with the peak representing the maximum rates of DNA synthesis, and typically found as a broad zone in mid-S phase. As seen in Figure 3B, the height of the peak was identical in *c-myc*^{+/+} and *-/-* cells, indicating that both the number of replication centers as well as the rates of DNA synthesis were equivalent in the two cell lines. In fact, the only significant difference between the arc profiles was that the *c-myc*^{-/-} arc was comprised of far fewer events. These data indicate that although the number of S-phase cells is clearly lower in *c-myc*^{-/-} than in *c-myc*^{+/+} cultures, S-phase progression is very similar in both.

Viewed in aggregate, the above-mentioned cell cycle analyses indicate that G₁ progression is very significantly compromised in *c-myc*^{-/-} cells, G₂ is clearly affected but to a much lesser degree, and S phase seems largely normal (Table 3). G₁ phase can be further subdivided into a postmitotic

Figure 3. Immunofluorescence and flow cytometric analysis of BrdU incorporation. (A) Immunofluorescence. Cells were pulsed for 5 min with 1 μ g/ml BrdU (3.2 μ M). (B) Flow cytometry. Cells were pulsed for 5 min. with 1 μ g/ml BrdU (3.2 μ M) and processed as described in MATERIALS AND METHODS.

Table 3. Aggregate summary of cell cycle durations

	<i>c-myc</i> ^{+/+}	<i>c-myc</i> ^{-/-}
G ₁ (h)	5	23
S (h)	11	13
G ₂ /M (h)	3	7
Total cell cycle (h)	19	43

phase (G₁-pm) and a pre-DNA-synthetic interval (G₁-ps), the two being clearly demarcated by the restriction (R) point at which cells become committed to continue the cell cycle without further mitogenic stimulation (Zetterberg *et al.*, 1995). To further delineate the G₁ phase defect in *c-myc*^{-/-} cells, cultures were synchronized at the beginning of G₁ by the mitotic shake-off procedure, serum was withdrawn at successive time points, and entry into S phase was monitored using BrdU labeling. As shown in Figure 4A, *c-myc*^{+/+} cells passed the R point very rapidly, the half-point of the transition to serum independence occurring ~1.5 h after mitotic shake-off. In contrast, 50% of *c-myc*^{-/-} cells passed the R point only 8 h after the shake-off. This 5.3-fold defect in progression through the R point is very similar in magnitude to the four- to fivefold elongation of G₁ phase seen in *c-myc*^{-/-} cells (Table 3); however, the 6.5-h G₁-pm delay cannot fully account for the overall 18-h G₁-phase elongation. Indeed, when S-phase entry data are superimposed on the restriction point profiles it becomes apparent that the post-R point G₁-ps interval is also significantly elongated in *c-myc*^{-/-} cells. If passage through the R point is expressed as a fraction of the total M- to S-phase interval, it can be seen that the R point occurs approximately one-third of the way through G₁ in both *c-myc*^{+/+} and ^{-/-} cells. In summary, the elongation of G₁ phase in *c-myc*^{-/-} cells is due to defects in passage through the R point as well as the subsequent progression toward S phase.

Although we did not use drugs to synchronize cells in M phase, the shake-off procedure could introduce perturbations. We thus examined the relationship of the R point to S-phase entry in a different physiological setting, namely, synchronization in G₀ phase by a combination of contact inhibition and serum deprivation, and subsequent release into the cell cycle by dilution into serum-containing medium. As shown in Figure 4D,E, both *c-myc*^{+/+} and *c-myc*^{-/-} passed the R point at approximately two-thirds (58–60%) of the total S-phase progression time. The half point of S-phase entry was at 16.8 h and 22 h for *c-myc*^{+/+} and *c-myc*^{-/-} cells, respectively. We have previously measured S phase entry for *c-myc*^{-/-} cells at 32 h under conditions where quiescent cells were stimulated with serum but not trypsinized and reseeded at the time of stimulation. The additional relief of contact inhibition results in a shortening of the G₀→S transition in *c-myc*^{-/-} cells but has little or no effect on *c-myc*^{+/+} cells (Figure 6C); nevertheless, the delays in S-phase entry as well as in passage through the R point in *c-myc*^{-/-} cells are clearly discernible.

While this work was in progress, a report by Eick and colleagues (Holzer *et al.*, 2001) presented data that *c-myc*^{-/-} cultures are a mixture of very slowly cycling (or even non-cycling) cells and cells that cycle at essentially normal (*c-myc*^{+/+}) rates. Because our data presented above are not

compatible with that interpretation, we wished to address possible confounding factors. Because we had relied extensively on BrdU labeling methods, we examined whether BrdU incorporation into cellular DNA could have affected cell cycle progression under our experimental conditions. BrdU and its derivatives have been documented to perturb the cell cycle and activate p53 (Rieber *et al.*, 1996; Michishita *et al.*, 1999; Peng *et al.*, 2001; Suzuki *et al.*, 2001), but these effects have been associated with exposures to higher concentrations of BrdU and for longer times than the conditions used herein. We labeled *c-myc*^{+/+} and *c-myc*^{-/-} cells for 48 h with BrdU, harvested the cells, stained them with propidium iodide, and subjected them to a flow cytometric analysis of cell cycle distribution. No differences were apparent compared with the cell cycle profiles of unlabeled, exponentially cycling control cells (our unpublished data). BrdU has been reported to induce both p53-dependent and p53-independent arrests (Rieber *et al.*, 1996; Peng *et al.*, 2001), but the induction of p21^{Cip1/Waf1} gene expression seems to be a universal hallmark of the BrdU response (Rieber *et al.*, 1996; Suzuki *et al.*, 2001). We therefore analyzed BrdU-labeled cells by real-time quantitative PCR for changes in p21 mRNA abundance (Figure 5A); again, no changes were observed. It thus seems that BrdU labeling under the conditions used herein does not elicit cell cycle perturbations.

We wished to further examine, using a completely distinct method of analysis, whether *c-myc*^{-/-} cultures consist of separate populations of cycling and noncycling cells. To this end, we used a method that measures the dilution of the vital dye CFSE. CFSE is a fluorescent dye that penetrates cell membranes and is metabolized and trapped within cells. Because CFSE is evenly distributed to daughter cells, the fluorescence intensity decreases by half with each cell division. This method has been widely used in immunology (Lyons, 1999) and neurobiology (Groszer *et al.*, 2001) to track cells both in vitro and in vivo for up to 10 generations. Dye dilution was found to be completely uniform for both *c-myc*^{+/+} and *c-myc*^{-/-} cells (Figure 5, B and C). Cohorts of noncycling (or slowly cycling) cells would be visualized as discrete peaks (or shoulders) at higher fluorescence intensity values. However, the peaks were found to be symmetrical and of the same width in both cultures at all time points. Furthermore, the rate of dye dilution (decrease in fluorescence intensity as a function of time) was consistent with the doubling times measured by standard growth curves. We therefore conclude that under our exponential growth conditions both *c-myc*^{+/+} and *c-myc*^{-/-} cultures are uniformly composed of continuously cycling cells.

We subsequently sought possible explanations for the discrepancy between our data and that reported by Holzer *et al.* (2001). Their study used time lapse photography of cells seeded at relatively high densities; it is thus conceivable that *c-myc*^{-/-} cells are capable of a limited growth spurt under these conditions, perhaps due to effects such as conditioning of the medium, deposition of extracellular matrix, or even transient cell-cell contact. To address these possibilities *c-myc*^{+/+} and ^{-/-} cells were seeded at low density, propagated continuously with frequent media changes until growth arrest due to high density was reached, and monitored at regular intervals for cell number as well as BrdU incorporation. As shown in Figure 6A, both cultures grew at constant rates and leveled off at equivalent cell densities

Figure 4. Determination of the G₁ restriction point. Cells were synchronized by mitotic shake-off (A-C), serum starvation (D-F), or contact inhibition (G-I) and replated in 10% CS. At successive time points serum stimulation was withdrawn and cells were processed for BrdU incorporation as described in MATERIALS AND METHODS. Temporal representations of the M- to S-phase interval and of the G₀-to-S-phase interval are shown in C,F,I; the time intervals in all panels are drawn on the same scale. The labeling indices (means $\pm$ SD, n = 11–13 fields/time point) denote BrdU-positive cells as percentage of total cells. A minimum of 562 *c-myc*^{+/+} and 746 *c-myc*^{-/-} cells/time point were scored. The S-phase entry data shown in A and B were overlaid from Figure 2A. The BrdU labeling indices in this figure were obtained using in situ immunohistochemistry methods.

Figure 5. Effect of BrdU labeling on p21^{Cip1} mRNA expression (A) and long-term monitoring of cell proliferation by using CFSE dye dilution. (A) Cells were labeled with BrdU, RNA was harvested, and analyzed by qualitative PCR as described in MATERIALS AND METHODS. p21 expression was normalized to GAPDH. (B) CFSE dye dilution. Live cells were briefly labeled with CFSE in suspension as described in MATERIALS AND METHODS and immediately replated at subconfluent conditions in complete medium. The $t = 0$ value was obtained from an aliquot of cells immediately after labeling. Flow cytometric analysis was done on live cells.

($\sim 1 \times 10^5$ cells/cm²). BrdU incorporation remained relatively constant throughout the exponential phase (Figure 6B) at close to previously observed values. It is noteworthy that if the culture medium was replenished at less frequent intervals (4 d instead of 2 d) *c-myc*^{-/-} cultures reached two- to threefold lower saturation densities than *c-myc*^{+/+} cultures (our unpublished data). It thus seems that the greater sensitivity of *c-myc*^{-/-} cultures to contact inhibition reported by Holzer *et al.* (2001) was due to suboptimal culture conditions, most likely medium depletion. It is also noteworthy that the cycling rates of *c-myc*^{+/+} cells in the Holzer *et al.* (2001) study were uncharacteristically slow (28 h).

In our search for conditions that could at least partially equalize the proliferation rates of *c-myc*^{+/+} and *c-myc*^{-/-} cells, we noticed that in the experiment to determine the R point during the G₀→S transition by using serum-starved and restimulated cells (Figure 4B), a significant fraction (up

to 30%) of *c-myc*^{-/-} cells entered S phase with kinetics that matched the fastest *c-myc*^{+/+} cells. To further explore this phenomenon, we used another method of G₀ synchronization, namely, contact inhibition without concomitant serum deprivation. In most cell lines (including Rat-1) this method is known to yield inferior G₀ arrest compared with serum deprivation, and is thus seldomly used. To our surprise, when contact inhibited cells were released into the cycle (Figure 4C), *c-myc*^{+/+} and *c-myc*^{-/-} cultures entered S phase at equivalent times (*c-myc*^{-/-} cells were even somewhat faster). Both *c-myc*^{+/+} and *c-myc*^{-/-} cells were affected by this regimen: relative to synchronization by serum deprivation, *c-myc*^{+/+} cells were slowed down and *c-myc*^{-/-} cells were accelerated. It was especially striking that these differences were largely caused by changes in the duration of G₁-ps, which was lengthened in *c-myc*^{+/+} cells and dramatically shortened in *c-myc*^{-/-} cells, such that they

Figure 6. Influence of cell density on proliferation (A) BrdU incorporation (B) and kinetics of S-phase entry from G₀ (C). (A) Growth curves for *c-myc*^{+/+} and *c-myc*^{-/-} cells were generated under normal and low (2%) O₂ conditions as indicated in MATERIALS AND METHODS. Dashed horizontal lines denote cell densities of ~ 50 and 100% confluence. (B) BrdU incorporation for experiments shown in A. At selected time points duplicate plates were withdrawn from the growth curve regimens and pulse labeled with BrdU. The labeling indices (means $\pm$ SEM, $n = 10$ [*c-myc*^{+/+}] and $n = 11$ [*c-myc*^{-/-}] fields/time point) denote BrdU-positive cells as percentage of total cells. A minimum of 625 (*c-myc*^{+/+}) and 499 (*c-myc*^{-/-}) cells/time point were scored. (C) G₀-to-S transition. Cells were made quiescent by serum deprivation as indicated in MATERIALS AND METHODS. Cell cycle entry was initiated by trypsinizing the cells and reseeding at subconfluent density in 10% CS-supplemented medium (reseeded) or simply changing the medium (not reseeded). The BrdU labeling indices in B were obtained using in situ immunohistochemistry methods. S-phase entry data in C was obtained by flow cytometry.

Figure 7. Total cellular protein determination by using sulforhodamine labeling and flow cytometric analysis. Culture conditions to obtain exponential phase (A), serum-starved (B), and contact-inhibited (C) cells are indicated in MATERIALS AND METHODS. Cells were harvested by trypsinization, stained with SR, and analyzed by flow cytometry. The experiment was repeated on at least three separate occasions; a representative data set is shown. Protein content of exponentially cycling *c-myc*^{+/+} and *c-myc*^{-/-} cells is very similar and in some experiments the two peaks were completely overlapping. The histograms shown were carefully gated on single whole cells (by using a FL2-W × FL2-A dot plot) to exclude aggregates and debris.

entered S phase almost immediately after passing the R point. In contrast, the interval from G₀ to the R point was relatively unaffected in each cell line by the different synchronization regimens.

Because duration of G₁-ps is not affected by mitogens but is the cell cycle interval characterized by accumulation of mass, we examined cell size under our growth and G₀ arrest conditions. Although such determinations are frequently made by measuring forward scatter in a flow cytometer, we found this method to be unreliable. We believe this is because forward scatter is affected by parameters such as cell shape, surface properties, and internal structure. We therefore used a flow cytometric method that measures total cellular protein by using the general protein dye sulforhodamine 101 (Engelhard, 1997). Exponentially cycling *c-myc*^{+/+} and *c-myc*^{-/-} cells had very similar protein content (Figure 7A), which is consistent with previous data showing that they have equivalent cell size (Mateyak *et al.*, 1997). Serum-starved cells became smaller, as previously reported (Larson *et al.*, 1986), although this effect was much less pronounced in *c-myc*^{-/-} cells (Figure 7B). In addition, *c-myc*^{-/-} cultures accumulated significant numbers of larger cells, as indicated by a prominent shoulder on the high intensity side of the peak (the data were carefully gated on single cells to exclude aggregates). The difference in cell size between *c-myc*^{+/+} and *c-myc*^{-/-} cells was even more pronounced if cultures were allowed to reach confluence in the presence of full-serum supplementation (Figure 7C).

A simple explanation for the observed rapid S-phase entry of *c-myc*^{-/-} cells (Figure 4C) is that rates of G₁-ps progression are strongly influenced by the size of the starting cell. In other words, large cells need a minimal G₁-ps to reach critical size for progression into S phase. Although we have not followed cells all the way from G₀ into mitosis, our data that G₁ accounts for most of the difference in cell cycle duration between *c-myc*^{+/+} and *c-myc*^{-/-} cells, as well as other studies showing that most cell cycle variability is due to changing lengths of G₁-ps (Zetterberg and Larsson, 1991), would predict that *c-myc*^{-/-} cells are capable of relatively fast cell cycle transit times under some conditions. Furthermore, we have shown that rapid cell cycle transit is correlated with

large cell mass, and that cell mass is positively correlated with high-density culture in the presence of full serum supplementation. We have observed that these conditions arise if cultures that are not continuously maintained at subconfluent conditions, especially with *c-myc*^{-/-} cells that tend to aggregate in larger islands and patches.

DISCUSSION

The series of labeling experiments presented in this communication show that the reduced proliferation rate previously documented for *c-myc*^{-/-} cells is due primarily due to a very significant lengthening of G₁ phase (four- to fivefold) as well as a more minor lengthening of G₂ phase (twofold). S-phase duration was found to be largely unaffected. G₁ phase is comprised of a postmitotic interval (G₁-pm) and a pre-DNA-synthetic interval (G₁-ps), the two being separated by the restriction (R) point (Zetterberg *et al.*, 1995). The second significant finding of this study is that both the G₁-pm and G₁-ps intervals were equally compromised in *c-myc*^{-/-} cells. Finally, we showed that the majority of cells in *c-myc*^{-/-} cultures during asynchronous, exponential phase growth display uniform cell cycles; in other words, no evidence was found for the existence of differentially cycling cohorts of cells.

Data from all experiments have been combined (Table 3) to derive what we consider to be the best approximations of cell cycle phase durations, using the following considerations. First, all methods, including direct labeling after mitotic shake-off, gave G₁ phase durations that were in close agreement for both cell lines. Second, although flow cytometric quantification of BrdU incorporation did not pick up significant differences between *c-myc*^{+/+} and *c-myc*^{-/-} cells, kinetic labeling methods showed a slight S-phase lengthening in *c-myc*^{-/-} cells. Therefore, S-phase durations were adjusted to 11 and 13 h for *c-myc*^{+/+} and *c-myc*^{-/-} cells, respectively. Although probably real, this difference is very small (1.2-fold) with respect to overall S-phase duration. Third, the kinetic determination of G₂ phase using the labeled mitoses method was considered the most direct and

reliable, and these values were thus used preferentially. Fourth, overall cell cycle durations were set to 19 and 43 h for *c-myc*^{+/+} and *c-myc*^{-/-} cells, respectively, and individual cell cycle phases were adjusted accordingly. These values were used because they were consistently observed with the batch of serum used in the experiments reported herein. It should be noted, however, that proliferation can be very sensitive to culture conditions, and that over several years we have observed growth rates in the range of 17–23 h and 42–60 h for *c-myc*^{+/+} and *c-myc*^{-/-} cells, respectively.

Under most conditions normal mammalian cells exhibit a state of balanced growth such that cellular mass (mostly protein) doubles exactly during each cell cycle (Zetterberg and Larsson, 1991). It is commonly observed that cells can adjust cell cycle progression in response to changes in growth (accumulation of mass) to maintain a constant size at the next cell division. Because rates of protein synthesis can change dramatically in response to many environmental conditions, the maintenance of a relatively constant cell size during proliferation under changing conditions is an indication of the existence of cell size monitoring processes. Experiments showing that growth and proliferation could be uncoupled under some conditions led to proposals for distinct “protein” and “chromosome” cell cycles (Baserga, 1984). For example, because withdrawal of mitogenic stimulation results in a drop in protein synthesis, cells that have passed the restriction point will progress to mitosis and divide at a smaller size than normal. Although the mechanisms that coordinate cell growth and cell cycle progression in mammalian cells are poorly understood, it has become apparent that the G₁ phase interval between the restriction point and S phase (G₁-ps) can vary significantly in length under different growth conditions (and also between different cells in the same culture) and may be the primary point at which adjustments in cell cycle progression can be made in response to variations in cell mass (Ekholm *et al.*, 2001).

c-Myc has been associated with promoting cell proliferation as well as cell growth. Under some conditions Myc is sufficient in triggering progression into S phase (Eilers *et al.*, 1991), whereas others have reported that Myc acts as a immediate-early competence factor that cooperates with platelet-poor plasma (Kaczmarek *et al.*, 1985). Both effects are best explained by direct effects on cell cycle progression. On the other hand, several recent examples point to an important, perhaps primary, role for c-Myc in promoting macromolecular synthesis and accumulation of cell mass (Iritani and Eisenman, 1999; Johnston *et al.*, 1999; Beier *et al.*, 2000; Greasley *et al.*, 2000; Kim *et al.*, 2000). The observation that rRNA and protein synthesis are threefold slower in *c-myc* null rat fibroblasts yet the cells maintain normal size (Mateyak *et al.*, 1997) can be explained by the existence of cell size checkpoints that slow cell cycle progression during balanced growth.

The fact that loss of c-Myc causes a significant lengthening of G₁-ps is most consistent with a role of c-Myc in cell growth. Progression through this cell cycle interval is independent of mitogenic signaling (Pardee, 1989) and is the time when most of the macromolecular synthesis in preparation for S phase takes place (Zetterberg *et al.*, 1995). Not surprisingly, ribosomal biogenesis is rapid during G₁-ps (Grummt, 1999). *c-myc*^{-/-} cells accumulate rRNA at a reduced rate (Mateyak *et al.*, 1997), and both ribosomal pro-

cessing factors and translation initiation factors have been reported to be transcriptional targets of c-Myc (Schmidt, 1999; Coller *et al.*, 2000; Greasley *et al.*, 2000). Neither ribosomal biogenesis nor rates of translation have been directly examined in *c-myc*^{-/-} cells, however, the overall rate of protein synthesis was found to be reduced by some threefold (Mateyak *et al.*, 1997). Although the length of G₁-ps would be expected to be strongly affected by rates of protein synthesis, a variety of metabolic enzymes have also been implicated as c-Myc targets (Dang, 1999; Coller *et al.*, 2000; Guo *et al.*, 2000) and may well play a role in G₁-ps progression.

On the other hand, the observed defect in progression through the R point is more consistent with a direct role of c-Myc on the cell cycle machinery. Several cell cycle regulators active in early G₁ phase, including p27 (Mateyak *et al.*, 1999; Yang *et al.*, 2001), gadd45 (Marhin *et al.*, 1997; Bush *et al.*, 1998), cyclin D2 (Bouchard *et al.*, 1999), and Cdk4 (Hermeking *et al.*, 2000) have been implicated as transcriptional targets of c-Myc. Indeed, the earliest known defect in cell cycle progression in *c-myc*^{-/-} cells is a very significant (10- to 15-fold) reduction in cyclin D-Cdk4/6 activation (Mateyak *et al.*, 1999; Obaya *et al.*, 2002). Although passage through the R point is dependent on protein synthesis (Pardee, 1989), rapid ribosome biogenesis and protein synthesis does not occur until later in G₁. Because cell mass does not increase during G₁-pm (Zetterberg and Larsson, 1991), it can be argued that defects in cell growth caused by lack of c-Myc would be unlikely to severely affect progression up to the R point. However, the role of c-Myc in R point progression requires closer scrutiny. First, reports of the competence-promoting activity of c-Myc were based on overexpression and may not reflect a physiological function. Second, although rRNA synthesis is not up-regulated until G₁-ps (Ciarmatori *et al.*, 2001; Voit and Grummt, 2001), protein synthesis at early times in G₁ has not been directly compared between *c-myc*^{+/+} and *-/-* cells. Thus, it is possible that reduced translation in *c-myc*^{-/-} cells may play a role in R point progression.

Several lines of evidence point to multiple roles for c-Myc in cell cycle progression. Perhaps the most striking is the finding that *c-myc* null cells have never been observed to revert to faster growth, and concerted efforts to identify downstream targets by functional complementation with retroviral cDNA libraries have been uniformly unsuccessful (Berns *et al.*, 2000; Nikiforov *et al.*, 2000). We show herein that loss of c-Myc results predominantly in a G₁ phase defect, and that progression through G₁-pm and G₁-ps are equally affected. These results are best explained by a model in which c-Myc directly affects cell growth (accumulation of mass) and cell proliferation (the cell cycle machinery) by independent pathways. Recent results that implicate both some metabolic enzymes and cell cycle regulators as direct transcriptional targets of c-Myc are consistent with this hypothesis.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Jennifer Rosenberg. This project was supported by a National Institutes of Health research grant GM-41690 (to J.M.S.).

REFERENCES

- Amati, B., Frank, S.R., Donjerkovic, D., and Taubert, S. (2001). Function of the c-Myc oncoprotein in chromatin remodeling and transcription. *Biochim. Biophys. Acta* 1471, M135–M145.
- Amati, B., and Land, H. (1994). Myc-Max-Mad: a transcription factor network controlling cell cycle progression, differentiation and death. *Curr. Opin. Genet. Dev.* 4, 102–108.
- Baserga, R. (1984). Growth in size and cell DNA replication. *Exp. Cell Res.* 151, 1–5.
- Beier, R., Burgin, A., Kiermaier, A., Fero, M., Karsunky, H., Saffrich, R., Moroy, T., Ansorge, W., Roberts, J., and Eilers, M. (2000). Induction of cyclin E-cdk2 kinase activity, E2F-dependent transcription and cell growth by Myc are genetically separable events. *EMBO J.* 19, 5813–5823.
- Berezney, R., Dubey, D.D., and Huberman, J.A. (2000). Heterogeneity of eukaryotic replicons, replicon clusters, and replication foci. *Chromosoma* 108, 471–484.
- Berns, K., Hijmans, E.M., Koh, E., Daley, G.Q., and Bernards, R. (2000). A genetic screen to identify genes that rescue the slow growth phenotype of c-myc null fibroblasts. *Oncogene* 19, 3330–3334.
- Blackwood, E.M., and Eisenman, R.N. (1991). Max: A helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with myc. *Science* 251, 1211–1217.
- Bouchard, C., Thieke, K., Maier, A., Saffrich, R., Hanley-Hyde, J., Ansorge, W., Reed, S., Sicinski, P., Bartek, J., and Eilers, M. (1999). Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. *EMBO J.* 18, 5321–5333.
- Bush, A., Mateyak, M.K., Dugan, K., Obaya, A., Adachi, S., Sedivy, J.M., and Cole, M.D. (1998). c-myc null cells misregulate *cad* and *gadd45* but not other proposed c-Myc targets. *Genes Dev.* 12, 3797–3802.
- Cheng, S.W., Davies, K.P., Yung, E., Beltran, R.J., Yu, J., and Kalpana, G.V. (1999). c-MYC interacts with INI1/hSNF5 and requires the SWI/SNF complex for transactivation function. *Nat. Genet.* 22, 102–105.
- Ciarmatori, S., Scott, P.H., Sutcliffe, J.E., McLees, A., Alzuheri, H.M., Dannenberg, J.H., te-Riele, H., Grummt, I., Voit, R., and White, R.J. (2001). Overlapping functions of the pRb family in the regulation of rRNA synthesis. *Mol. Cell. Biol.* 21, 5806–5814.
- Claassen, G., and Hann, S.R. (1999). Myc-mediated transformation: the repression connection. *Oncogene* 18, 2925–2933.
- Coller, H.A., Grandori, C., Tamayo, P., Colbert, T., Lander, E.S., Eisenman, R.N., and Golub, T.R. (2000). Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. *Proc. Natl. Acad. Sci. USA* 97, 3260–3265.
- Dang, C.V. (1999). c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Mol. Cell. Biol.* 19, 1–11.
- Davis, A.C., Wims, M., Spotts, G.D., Hann, S.R., and Bradley, A. (1993). A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. *Genes Dev.* 7, 671–682.
- Eberhardy, S.R., and Farnham, P.J. (2001). c-Myc mediates activation of the *cad* promoter via a post-RNA polymerase II recruitment mechanism. *J. Biol. Chem.* 276, 48562–48571.
- Eilers, M., Schirm, S., and Bishop, J.M. (1991). The MYC protein activates transcription of the alpha-prothymosin gene. *EMBO J.* 10, 133–141.
- Ekholm, S.V., Zickert, P., Reed, S.I., and Zetterberg, A. (2001). Accumulation of cyclin E is not a prerequisite for passage through the restriction point. *Mol. Cell. Biol.* 21, 3256–3265.
- Elend, M., and Eilers, M. (1999). Cell growth: downstream of Myc - to grow or to cycle? *Curr. Biol.* 9, R936–R938.
- Engelhard, H.H. (1997). Flow cytometric applications of sulforhodamine 101 as a fluorescent stain for total cellular protein. *Biotech. Histochem.* 72, 1–9.
- Facchini, L.M., Chen, S., Marhin, W.W., Lear, J.N., and Penn, L.Z. (1997). The Myc negative autoregulation mechanism requires Myc-Max association and involves the c-myc P2 minimal promoter. *Mol. Cell. Biol.* 17, 100–114.
- Frank, S.R., Schroeder, M., Fernandez, P., Taubert, S., and Amati, B. (2001). Binding of c-Myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. *Genes Dev.* 15, 2069–2082.
- Gallant, P., Shio, Y., Cheng, P.F., Parkhurst, S.M., and Eisenman, R.N. (1996). Myc and Max homologs in *Drosophila*. *Science* 274, 1523–1527.
- Grandori, C., Cowley, S.M., James, L.P., and Eisenman, R.N. (2000). The Myc/Max/Mad network and the transcriptional control of cell behavior. *Annu. Rev. Cell Dev. Biol.* 16, 653–699.
- Gratzner, H. (1982). Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science* 218, 474–5.
- Greasley, P.J., Bonnard, C., and Amati, B. (2000). Myc induces nucleolin and BN51 genes: possible implications in ribosome biogenesis. *Nucleic Acids Res.* 28, 446–453.
- Groszer, M., Erickson, R., Scripture-Adams, D.D., Lesche, R., Trumpp, A., Zack, J.A., Kornblum, H.I., Liu, X., and Wu, H. (2001). Negative regulation of neural stem/progenitor cell proliferation by the *Pten* tumor suppressor gene in vivo. *Science* 294, 2186–2189.
- Grummt, I. (1999). Regulation of mammalian ribosomal gene transcription by RNA polymerase I. *Prog. Nucleic Acid Res. Mol. Biol.* 62, 109–154.
- Guo, Q.M., Malek, R.L., Kim, S., Chiao, C., He, M., Ruffy, M., Sanka, K., Lee, N.H., Dang, C.V., and Liu, E.T. (2000). Identification of c-myc responsive genes using rat cDNA microarray. *Cancer Res.* 60, 5922–5928.
- Henriksson, M., and Luscher, B. (1996). Proteins of the Myc network: essential regulators of cell growth and differentiation. *Adv. Cancer Res.* 68, 109–182.
- Hermeking, H., *et al.* (2000). Identification of CDK4 as a target of c-MYC. *Proc. Natl. Acad. Sci. USA* 97, 2229–2234.
- Holzer, M., Kohlhuber, F., Schlosser, I., Holzer, D., Luscher, B., and Eick, D. (2001). Myc/Max/Mad regulate the frequency but not the duration of productive cell cycles. *EMBO Rep.* 2, 1125–1132.
- Iritani, B.M., and Eisenman, R.N. (1999). c-Myc enhances protein synthesis and cell size during B lymphocyte development. *Proc. Natl. Acad. Sci. USA* 96, 13180–13185.
- Johnston, L.A., Prober, D.A., Edgar, B.A., Eisenman, R.N., and Gallant, P. (1999). *Drosophila* myc regulates cellular growth during development. *Cell* 98, 779–790.
- Kaczmarek, L., Hyland, J.K., Watt, R., Rosenberg, M., and Baserga, R. (1985). Microinjected c-Myc as a competence factor. *Science* 228, 1313–1315.
- Kim, S., Li, O., Dang, C.V., and Lee, L.A. (2000). Induction of ribosomal genes and hepatocyte hypertrophy by adenovirus-mediated expression of c-Myc in vivo. *Proc. Natl. Acad. Sci. USA* 97, 11198–11202.

- Larsson, O., Dafgard, E., Engstrom, W., and Zetterberg, A. (1986). Immediate effects of serum depletion on dissociation between growth in size and cell division in proliferating 3T3 cells. *J. Cell. Physiol.* 127, 267–273.
- Luscher, B., and Larsson, L.G. (1999). The basic region/helix-loop-helix/leucine zipper domain of Myc proto-oncogenes: function and regulation. *Oncogene* 18, 2955–2966.
- Lyons, A.B. (1999). Divided we stand: tracking cell proliferation with carboxyfluorescein diacetate succinimidyl ester. *Immunol. Cell Biol.* 77, 509–515.
- Marhin, W.W., Chen, S., Facchini, L.M., Fornace, A.J., Jr., and Penn, L.Z. (1997). Myc represses the growth arrest gene gadd45. *Oncogene* 14, 2825–2834.
- Mateyak, M.K., Obaya, A.J., Adachi, S., and Sedivy, J.M. (1997). Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. *Cell Growth Differ.* 8, 1039–1048.
- Mateyak, M.K., Obaya, A.J., and Sedivy, J.M. (1999). c-Myc regulates cyclin D-Cdk4 and -Cdk6 activity but affects cell cycle progression at multiple independent points. *Mol. Cell. Biol.* 19, 4672–4683.
- McMahon, S.B., Van Buskirk, H.A., Dugan, K.A., Copeland, T.D., and Cole, M.D. (1998). The novel ATM-related protein TRAP is an essential cofactor for the c-Myc and E2F oncoproteins. *Cell* 94, 363–374.
- McMahon, S.B., Wood, M.A., and Cole, M.D. (2000). The essential cofactor TRRAP recruits the histone acetyltransferase hGCN5 to c-Myc. *Mol. Cell. Biol.* 20, 556–562.
- Michishita, E., Nakabayashi, K., Suzuki, T., Kaul, S.C., Ogino, H., Fujii, M., Mitsui, Y., and Ayusawa, D. (1999). 5-bromodeoxyuridine induces senescence-like phenomena in mammalian cells regardless of cell type or species. *Biochem. J.* 126, 1052–1059.
- Moscovitis, G., and Pardee, A.B. (1980). Citric acid arrest and stabilization of nucleoside incorporation into cultured cells. *Anal Biochem* 101, 221–224.
- Nesbit, C.E., Tersak, J.M., Grove, L.E., Drzal, A., Choi, H., and Prochownik, E.V. (2000). Genetic dissection of c-myc apoptotic pathways. *Oncogene* 19, 3200–3212.
- Nikiforov, M.A., Kotenko, I., Petrenko, O., Beavis, A., Valenick, L., Lemischka, I., and Cole, M.D. (2000). Complementation of Myc-dependent cell proliferation by cDNA expression library screening. *Oncogene* 19, 4828–4831.
- O'Hagan, R.C., Ohh, M., David, G., de Alboran, I.M., Alt, F.W., Kaelin, W.G.J., and DePinho, R.A. (2000). Myc-enhanced expression of Cul1 promotes ubiquitin-dependent proteolysis and cell cycle progression. *Genes Dev.* 14, 2185–2191.
- Obaya, A.J., Kotenko, I., Cole, M.D., and Sedivy, J.M. (2002). The proto-oncogene *c-myc* acts through the cyclin-dependent kinase (Cdk) inhibitor p27^{Kip1} to facilitate the activation of Cdk4/6 and early G1 phase progression. *J. Biol. Chem.* 277, 31263–31269.
- Obaya, A.J., Mateyak, M.K., and Sedivy, J.M. (1999). Mysterious liaisons: the relationship between c-Myc and the cell cycle. *Oncogene* 18, 2934–2941.
- Oster, S.K., Ho, C.S.W., Soucie, E.L., and Penn, L.Z. (2002). The *myc* oncogene: Marvelously Complex. *Adv. Cancer Res.* 84, 81–154.
- Pardee, A.B. (1989). G1 events and regulation of cell proliferation. *Science* 246, 603–608.
- Peng, D.F., Sugihara, H., and Hattori, T. (2001). Bromodeoxyuridine induces p53-dependent and -independent cell cycle arrests in human gastric carcinoma cell lines. *Pathobiology* 69, 77–85.
- Prendergast, G.C., and Ziff, E.B. (1991). Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. *Science* 251, 186–189.
- Rieber, M.S., Welch, D.R., Miele, M.E., and Rieber, M. (1996). p53-independent increase in p21WAF1 and reciprocal downregulation of cyclin A and proliferating cell nuclear antigen in bromodeoxyuridine-mediated growth arrest of human melanoma cells. *Cell Growth Differ.* 7, 197–202.
- Schmidt, E.V. (1999). The role of c-myc in cellular growth control. *Oncogene* 18, 2988–2996.
- Schreiber-Agus, N., Stein, D., Chen, K., Goltz, J.S., Stevens, L., and DePinho, R.A. (1997). Drosophila Myc is oncogenic in mammalian cells and plays a role in the diminutive phenotype. *Proc. Natl. Acad. Sci. USA* 94, 1235–1240.
- Schuhmacher, E.V., Staeger, M.S., Pajic, A., Polack, A., Weidle, U.H., Bornkamm, G.W., Eick, D., and Kohlhuber, F. (1999). Control of cell growth by c-Myc in the absence of cell division. *Curr. Biol.* 9, 1255–1258.
- Shichiri, M., Hanson, K.D., and Sedivy, J.M. (1993). Effects of *c-myc* expression on proliferation, quiescence, and the G₀ to G₁ transition in nontransformed cells. *Cell Growth Differ.* 4, 93–104.
- Soucie, E.L., Annis, M.G., Sedivy, J., Filmus, J., Leber, B., Andrews, D.W., and Penn, L.Z. (2001). Myc potentiates apoptosis by stimulating Bax activity at the mitochondria. *Mol. Cell. Biol.* 21, 4725–4736.
- Staller, P., et al. (2001). Repression of p15INK4b expression by Myc through association with Miz-1. *Nat. Cell Biol.* 3, 392–399.
- Suzuki, T., Minagawa, S., Michishita, E., Ogino, H., Fujii, M., Mitsui, Y., and Ayusawa, D. (2001). Induction of senescence-associated genes by 5-bromodeoxyuridine in HeLa cells. *Exp. Gerontol.* 36, 465–474.
- Trump, A., Refaeli, Y., Oskarsson, T., Gasser, S., Murphy, M., Martin, G.R., and Bishop, J.M. (2001). c-Myc regulates mammalian body size by controlling cell number but not cell size. *Nature* 414, 768–773.
- Voit, R., and Grummt, I. (2001). Phosphorylation of UBF at serine 388 is required for interaction with RNA polymerase I and activation of rDNA transcription. *Proc. Natl. Acad. Sci. USA* 98, 13631–13636.
- Wood, M.A., McMahon, S.B., and Cole, M.D. (2000). An ATPase/helicase complex is an essential cofactor for oncogenic transformation by c-Myc. *Mol. Cell* 5, 321–330.
- Xiao, Q., Claassen, G., Shi, J., Sedivy, J.M., and Hann, S.R. (1998). Transactivation-defective c-MycS retains the ability to regulate growth and apoptosis. *Genes Dev.* 12, 3803–3808.
- Yang, W., et al. (2001). Repression of transcription of the p27(Kip1) cyclin-dependent kinase inhibitor gene by c-Myc. *Oncogene* 20, 1688–1702.
- Zetterberg, A., and Larsson, O. (1991). Coordination between cell growth and cell cycle transit in mammalian cells. *Cold Spring Harbor Symp. Quant. Biol.* 56, 137–147.
- Zetterberg, A., Larsson, O., and Wiman, K. (1995). What is the restriction point? *Curr. Opin. Cell Biol.* 7, 835–842.