Cell Cycle Regulation of the c-Myc Transcriptional Activation Domain

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The product of the *c-myc* gene (c-Myc) is a sequence-specific DNA-binding protein that has previously been demonstrated to be required for cell cycle progression. Here we report that the c-Myc DNA binding site confers cell cycle regulation to a reporter gene in Chinese hamster ovary cells. The observed transactivation was biphasic with a small increase in G₁ and a marked increase during the S-to-G₂/M transition of the cell cycle. This cell cycle regulation of transactivation potential is accounted for, in part, by regulatory phosphorylation of the c-Myc transactivation domain. Together, these data demonstrate that c-Myc may have an important role in the progression of cells through both the G₁ and G₂ phases of the cell cycle.

Evidence has been obtained indicating an important functional role for the c-Myc protein during cellular proliferation and differentiation (22, 70, 82). For many years the function of c-Myc has been poorly understood. However, significant progress has recently been made towards understanding molecular details about the biological actions of c-Myc. Thus, it is now known that c-Myc can function as a transcription factor and that it is a sequence-specific DNA-binding protein with a transactivation domain, a basic region, and dimerization motifs that are required for interaction with a partner protein, Max (15, 23, 60, 74, 91, 114). It has also been demonstrated that the functional form of the c-Myc protein is represented by the heterodimeric complex of c-Myc and Max (3, 4, 14). Recent studies have shown that the c-Myc partner protein Max can also interact with at least two other proteins: Mad and Mxi1 (7, 119).

Treatment of quiescent cells with growth factors results in an increase in c-Myc expression (62). However, in growing cells, c-Myc is expressed constitutively (28, 49, 94, 112, 116). The expression of Max is also constitutive during the cell cycle (14, 16, 61, 73, 88, 89). These observations suggest that the level of expression of the functional c-Myc-Max heterodimer is not regulated in growing cells. This conclusion has been confirmed by coimmunoprecipitation studies which demonstrate that the level of the c-Myc-Max complex is invariant during the cell cycle (16). Under these conditions of constitutive expression, the function of the c-Myc-Max complex could be regulated by competition for binding to c-Myc DNA-responsive elements (CACGTG) present within promoter sequences (4, 5, 7, 66, 119). Alternatively, functional regulation could occur by a mechanism involving a posttranslational modification, such as phosphorylation (54, 55). Indeed, the phosphorylation state of c-Myc has been shown to be regulated during the cell cycle (71).

It is known that c-Myc is a substrate for at least two protein kinases: casein kinase II (72) and MAP kinase (2). The role of casein kinase II phosphorylation of c-Myc has not been defined (107), but it has been established that this protein kinase can inhibit DNA binding by Max homodimers

(10). In contrast, phosphorylation of c-Myc at the MAP kinase site, Ser-62, positively regulates the c-Myc transactivation domain (45, 99, 100). A regulatory role for this phosphorylation is consistent with the observation that Ser-62 is the major site of phosphorylation of the c-Myc protein in vivo (45). Significantly, Ser-62 is not only a substrate for phosphorylation by MAP kinase but also a substrate for in vitro phosphorylation by the cyclin-dependent protein kinase p34^{cdc2} (99). Thus, although expression of c-Myc is constitutive during the cell cycle, it is possible that the function of c-Myc is subject to cell cycle-dependent regulation by phosphorylation.

Cells that express high levels of c-Myc generally exhibit reduced requirements for growth factors (6, 68, 104, 105) and exhibit higher growth rates (68, 79) characterized by a shortened length of the G₁ phase of the cell cycle (58). The expression of c-Myc can also overcome growth arrest (6, 44, 65). Thus, c-Myc is a critical protein that is required for normal cell cycle progression (95, 103). In contrast, exit from the cell cycle may require down-regulation of c-Myc expression. One example is provided by the action of transforming growth factor beta (TGF-β) to cause both growth arrest and c-Myc down-regulation (21, 83, 84). Treatment of cultured cells with antisense *c-myc* oligonucleotides or with TGF-β1 causes an inhibition of cellular proliferation by blocking entry of cells into S phase (50, 51, 83, 84). The observation that antisense *c-myc* can initiate cellular differentiation (51) suggests that c-Myc down-regulation may also be mechanistically relevant to the process of differentiation. Strong support for this hypothesis is provided by the demonstration that c-Myc overexpression can block terminal differentiation (8, 11, 24, 30, 39, 67, 75, 92). There are, however, several exceptions to the simple correlation between cellular proliferation or differentiation and the level of c-Myc expression (31, 32, 52, 56, 64). One example of the complex relationship between c-Myc expression and cellular proliferation is provided by the possible role of c-Myc during apoptosis (12, 37, 101).

Several lines of evidence indicate that c-Myc may have a role during the G₁ phase of the cell cycle (50, 58). For example, c-Myc has been shown to be sufficient to cause cells to enter S phase (6, 34, 35). Furthermore, the observation that c-Myc can overcome the G₁ growth arrest induced by Rb (42) demonstrates that c-Myc does act at the G₁ phase

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of the cell cycle. However, these data do not indicate that the functional role of c-Myc is restricted to G₁. Indeed, c-Myc may also function at other phases of the cell cycle (51). Consistent with this hypothesis is the observation that c-Myc can rescue a deficiency in BAF-BO3 cells which prevents progress of the cells beyond S phase after epidermal growth factor stimulation (103).

The purpose of this study was to evaluate the cell cycle regulation of c-Myc function as a transcription factor. We report that the level of transactivation oscillates as cells progress through the cell cycle and that there is a marked increase in transactivation during the S-to-G₂/M transition. The regulation of transactivation potential can be accounted for, in part, by changes in the phosphorylation state of c-Myc. Together, these data indicate that there may be an important function of c-Myc during the G₂ phase of the cell cycle.

MATERIALS AND METHODS

Materials. [³²P]phosphate and ¹²⁵I-protein A were from Dupont-New England Nuclear. [³H]thymidine was from ICN. Aphidicolin, hydroxyurea, and *o*-nitrophenyl-β-D-galactopyranoside were purchased from Sigma Chemical Co. (St. Louis, Mo.). Hoechst 33342 was from Calbiochem-Behring Corp. (La Jolla, Calif.). Fetal bovine serum (FBS) was from Gibco-BRL Inc. Luciferin was purchased from Analytical Luminescence Labs (San Diego, Calif.). The polyclonal rabbit anti-GAL4 antibody was a gift from M. Ptashne (Harvard University). The anti-Myc immunoglobulin G was purified from serum that was obtained after immunization of a sheep with a bacterially expressed human c-Myc protein (residues 2 to 243) as an antigen.

Plasmids. The plasmids pM21 and pRSV-Neo were obtained from C. V. Dang (Johns Hopkins School of Medicine) and C. W. Woon (University of Massachusetts Medical School), respectively. The plasmids pCH110 and pUC13 were from Pharmacia-LKB Biotechnology Inc. The activator plasmids pGAL4/Myc and pGAL4/[Ala⁶²]Myc were constructed by using the vector pSG424 and have been described previously (2). The plasmids pSG424-Luc and pG5E1bLuc were prepared by cloning the luciferase gene from pGEM-Luc (Promega Biotech) into the vectors pSG424 and pG5E1bCAT, respectively, as described previously (100).

The plasmids pMyc0E1bLuc and pMyc1E1bLuc were prepared from pG5E1bLuc. The GAL4/E1b promoter was removed from pG5E1bLuc as a *Pst*I and *Kpn*I fragment and replaced with a double-stranded 20mer oligonucleotide that contains the E1b promoter TATA element (5' GAGGGTAT ATAATGTGGTAC 3' and 5' CACATTATATACCCTCTG CA 3') to create pMyc0E1bLuc. A c-Myc binding site (underlined) was inserted at the *Hind*III site of pMyc0E1bLuc as a 26mer double-stranded oligonucleotide (5' AG CTTAAGTACCACGTTGGTCAACTA 3' and 5' AGCTT AGTTGACCACGTTGGTCACTTA 3') to obtain the plasmid pMyc1E1bLuc.

Cell culture and transfection assays. COS-7 cells were subcultured in Dulbecco's modified Eagle's medium supplemented with 5% FBS, and transfections were performed by the DEAE-dextran method as previously described (2).

CHO cells were maintained in Ham's F-12 medium supplemented with 5% FBS. Cells expressing GAL4/Myc or GAL4/[Ala⁶²]Myc activator protein together with the reporter plasmid (pG5E1bLuc) were obtained by cotransfecting 5 μg of activator plasmid and 5 μg of each plasmid

together with 2 μg of pRSVneo by the calcium phosphate technique. G418-resistant colonies were isolated and screened for the fusion protein expression and luciferase reporter activity. Cells were also cotransfected with pRSV-Neo and (i) pG5E1bLuc (to monitor the basal expression of the reporter plasmid), (ii) pSG424-Luc (to monitor the activity of the activator expression vector), and (iii) pMyc1E1bLuc (to monitor reporter gene expression conferred by the c-Myc DNA binding site CACGTG).

CHO cells transfected with control DNA (pUC13), or with the activator plasmid alone, gave no measurable luciferase activity. In cells expressing the reporter plasmid (pG5E1bLuc) alone, the level of luciferase activity detected was very low (10⁴ light units/10⁶ cells). This reflects the basal activity of the E1b promoter. Cotransfection of the activator and reporter plasmids resulted in cells that expressed a high level of luciferase activity (~10⁷ light units/10⁶ cells). Cells expressing the pMyc1E1bLuc reporter plasmid had an intermediate level of luciferase activity (10⁵ light units/10⁶ cells).

Cell synchronization. CHO cells were seeded in 100-mm dishes for luciferase assays, immunoblotting, and immunoprecipitation; 150-mm tissue culture dishes were used for fluorescence-activated cell sorter analysis; and 6-well multidish plates were used for the analysis of [³H]thymidine incorporation. Log-phase cultures were synchronized at the G₁/S boundary by treatment for 18 h with 1 μg of aphidicolin per ml or 1 mM hydroxyurea, washed three times with serum-free medium, transferred to complete medium for 10 h, and then growth arrested by incubation in medium containing aphidicolin or hydroxyurea for 18 h. Hydroxyurea arrests cells near the G₁/S boundary at a point distinct from aphidicolin arrest (1, 26, 46, 81, 118). To arrest cells in the G₂ phase, the cells were presynchronized at G₁/S by treatment with 1 mM hydroxyurea for 18 h, washed three times with serum-free medium, and then incubated for an additional 12 h in complete medium supplemented with 7.5 μg of Hoechst 33342 dye per ml (113). To examine the possibility of delayed toxicity associated with the multistep synchronization procedures, cell proliferation was monitored by total cell count with a hemocytometer.

Analysis of cell synchrony. The synchrony of cells was monitored by assessing the cellular DNA content by flow cytometry, using a Becton Dickinson FACScan. For flow cytometric analysis, 6 × 10⁶ cells were trypsinized, washed in phosphate-buffered saline, fixed in a solution of 95% cold ethanol, and stored at 4°C. The fixed cells were stained with propidium iodide as described previously (111). The fluorescence intensity of propidium iodide, which is proportional to the cellular DNA content, was measured and analyzed with PC Lysys software (B. Woda, University of Massachusetts Medical School). To determine the time of DNA synthesis in synchronized cell cultures, the incorporation of thymidine into DNA was measured by pulse-labeling with [³H]thymidine (1 μCi/ml) for 30 min in 2 ml of medium. The cells were washed twice with ice-cold Krebs-Ringer HEPES (KRH) buffer (128 mM NaCl, 5 mM KCl, 5 mM MgSO₄, 1.3 mM CaCl₂, 50 mM HEPES, pH 7.4) (HEPES is *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and then incubated in KRH supplemented with 2% trichloroacetic acid at 4°C for 12 h on a shaking platform. The cells were washed once with KRH-2% trichloroacetic acid, three times with KRH, and once with ice-cold methanol. The cells were air dried and then solubilized with 1 ml of 0.2 M NaOH for 60 min at room temperature. An aliquot (500 μl) was removed and mixed with 100 μl of 1 M HCl and 5 ml of scintillation fluid

(Optifluor; Packard). Radioactivity was measured in a Beckman scintillation counter.

Measurement of luciferase and β -galactosidase activity. The protein concentration in cell extracts was measured by the Bradford dye binding procedure (BioRad) with bovine serum albumin as a standard. Luciferase activity was measured by using 5 μ l of cell extract with an Analytical Luminescence Laboratory (San Diego, Calif.) model 2010 luminometer (99) and expressed as light units per milligram of protein. The activity of β -galactosidase in COS cells was measured by mixing 15 μ l of cell extract with 3 μ l of 0.1 M MgCl₂-4.5 M β -mercaptoethanol, 66 μ l of 4 mg of *o*-nitrophenyl- β -D-galactopyranoside per ml, and 216 μ l of 0.1 M sodium phosphate (pH 7.5). The incubations were performed at 37°C for 5 min or until a faint yellow color appeared. The reactions were stopped by adding 500 μ l of 1 M Na₂CO₃ to each tube, and the optical density at 420 nm was measured with a Pharmacia-LKB spectrophotometer.

Isolation of metabolically labeled phosphoproteins by immunoprecipitation. COS-7 cells in 100-mm dishes were trypsinized 18 h posttransfection. The cells were pooled, reseeded into two 100-mm dishes (one for Western blot [immunoblot] analysis and the second for metabolic labeling with inorganic [³²P]phosphate). The cells were then incubated in Dulbecco's modified Eagle's medium containing 5% FBS for 10 h. Hydroxyurea (1 mM) was added to the medium for 18 h. The arrested cells were washed three times with serum-free medium and then incubated in serum-supplemented medium. During the last 4 h of incubation, the cells were transferred to phosphate-free modified Eagle's medium (Flow Labs Inc.) containing 1% FBS and 2 mCi of [³²P]phosphate (Dupont-NEN) per ml. The cells were lysed, and the GAL4 fusion proteins were isolated by immunoprecipitation using an anti-GAL4 antibody and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (2).

Phosphopeptide mapping and phosphoamino acid analysis. Phosphorylated proteins were digested with tosylphenylalanyl chloromethyl ketone-treated trypsin in 25 mM *N*-ethylmorpholine (pH 8.0) as described previously (2). Phosphopeptide mapping was performed by two-dimensional separation on 100- μ m cellulose thin-layer plates (Machery-Nagel). The first dimension was electrophoresis using 30% (vol/vol) formic acid at 500 V for 2 h at 4°C. The second dimension was chromatography in the vertical direction using butan-1-ol-pyridine-acetic acid-water (75:50:15:60). The origin is located in the lower left corner of each peptide map. Phosphoamino acid analysis was performed by partial acid hydrolysis (1 h at 110°C in 6 M HCl) and thin-layer electrophoresis as described previously (2).

Analysis of the level of GAL4/Myc expression. Cells growing in 100-mm tissue culture dishes were washed once with phosphate-buffered saline and collected in 0.5 ml of the same buffer. The cells were pelleted at 3,000 $\times$ *g* for 3 min at 4°C and resuspended in 0.5 ml of 10 mM HEPES, pH 8.0-1.5 mM MgCl₂-10 mM KCl-5 mM dithiothreitol-0.5% Nonidet P-40-1 mM phenylmethylsulfonyl fluoride-10 μ g of leupeptin. After incubation for 1 min on ice, the lysate was then centrifuged at 3,000 $\times$ *g* for 3 min. The pellet was then resuspended in 100 μ l of 20 mM HEPES (pH 8.0)-25% glycerol-0.42 M NaCl-0.2 mM EDTA-1 mM dithiothreitol-1 mM phenylmethylsulfonyl fluoride-10 μ g of leupeptin per ml. The extract was incubated on ice for 30 min and then centrifuged at 12,000 $\times$ *g* for 10 min at 4°C. The supernatant was collected as nuclear extract and stored at -80°C.

For COS cells, 20 μ l of nuclear extract was subjected to

SDS-PAGE and the resolved proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp.). The membrane was then probed with a polyclonal rabbit anti-GAL4 antibody to detect the GAL4/Myc fusion proteins, and the immune complexes were visualized by using ¹²⁵I-protein A.

For CHO cells, the GAL4/Myc proteins were first immunoprecipitated from 400 μ l of nuclear extract by using a rabbit anti-GAL4 antibody and 20 μ l of protein A-Sepharose. The immunoprecipitate was subjected to SDS-PAGE, and the proteins were transferred onto an Immobilon-P membrane. The membrane was then probed with a polyclonal sheep anti-Myc antibody, and the immune complexes were visualized with rabbit anti-sheep immunoglobulin G coupled to horseradish peroxidase by the enhanced chemiluminescence detection method (Amersham International PLC).

RESULTS

Synchronization of the cell cycle. To investigate the cell cycle dependence of c-Myc function, we have employed cultures of synchronously growing cells. Inhibitors were used to induce growth arrest at specific stages of the cell cycle. Release of the cells from the growth arrest yielded cultures that maintained synchrony during the subsequent cell cycle. Several drugs that cause reversible growth arrest of cultured cells were used in this study. Hydroxyurea, an inhibitor of deoxyribonucleotide synthase (1), and aphidicolin, an inhibitor of DNA polymerase α (81), both inhibit DNA synthesis. These drugs were used to arrest cells at the G₁/S boundary and early S phase, respectively (1, 26, 46, 76, 81, 118). Hoechst 33372, an inhibitor of DNA topoisomerase II, arrests cells in G₂ (85, 113). These methods of growth arrest are reversible and yield synchronous cell populations.

Flow cytometry was used to determine cellular DNA content following propidium iodide staining (Fig. 1). [³H]thymidine incorporation was used as a measure of DNA synthesis (Fig. 2C). Following release of CHO cells from the growth arrest caused by hydroxyurea or aphidicolin, the cells were found to synchronously progress through the cell cycle. The synthesis of DNA, measured as [³H]thymidine incorporation, began within 15 min after the withdrawal of aphidicolin (data not shown) and 30 min following the withdrawal of hydroxyurea (Fig. 2C). A high level of [³H]thymidine incorporation was sustained for approximately 3 h and was markedly decreased by 6 h (Fig. 2C). Thus, DNA replication in S phase starts shortly after the withdrawal of hydroxyurea or aphidicolin and ends at approximately 6 h. Measurement of the average DNA content of the cells by flow cytometry confirmed this result (Fig. 1). After exit from S phase, the cells traversed G₂ until approximately 9 h following the withdrawal of hydroxyurea when the cells reached the G₂/M boundary. Mitotic cells were identified by microscopic analysis at 9 to 10 h following hydroxyurea withdrawal. Subsequent to mitosis, cells at the G₁ phase of the cell cycle were observed by flow cytometry (Fig. 1). For a small population of cells, exit from G₂/M was delayed (27, 33), but the majority of the cells reached the G₁/S boundary of the next cell cycle at approximately 18 to 20 h following the withdrawal of hydroxyurea (Fig. 1 and 2). Together, these data establish that a relatively synchronous population of CHO cells can be prepared for cell cycle analysis of the transactivation potential of c-Myc. A synchronous population of COS-7 cells was also obtained by the same procedures (data not shown).

FIG. 1. Cell cycle analysis of the DNA content of synchronized CHO cells. CHO cells were released from hydroxyurea-induced growth arrest at the G₁/S phase boundary and then harvested at defined times. The DNA content of the CHO cells was analyzed by flow cytometry and staining with propidium iodide. Histograms of the flow cytometric data obtained after different times of hydroxyurea withdrawal are presented.

Cell cycle-dependent expression of a reporter gene is conferred by the c-Myc DNA binding site CACGTG. A specific DNA binding site for c-Myc has been identified as the E-box sequence CACGTG (13, 48, 63, 80, 90). Therefore, to investigate the level of c-Myc-dependent transactivation of gene expression, we constructed a reporter plasmid (pMyc1E1bLuc) that consists of a site for c-Myc binding (CACGTG) located adjacent to a minimal promoter (TATA) element and the firefly luciferase gene (Fig. 2A). A similar experimental strategy designed to monitor c-Myc function by using a reporter plasmid containing sites for c-Myc binding has been reported previously (3, 5, 45, 66). Cells transfected with the reporter plasmid pMyc1E1bLuc ex-

pressed a high level of luciferase activity ($\sim 10^5$ light units/ 10^6 cells), and a three- to fivefold-increased level of luciferase activity was found when the cells were cotransfected with the c-Myc expression vector pM21. In contrast, cells transfected with a control reporter plasmid that lacked c-Myc binding sites (pMyc0E1bLuc) resulted in the expression of a low level of luciferase activity ($\sim 10^4$ light units/ 10^6 cells) that was unaltered by cotransfection of the cells with the c-Myc expression vector. Together, these data demonstrate that the reporter plasmid pMyc1E1bLuc can be used as a probe to investigate the function of c-Myc (and other E-box binding proteins) expressed in cultured cells.

To assess the cell cycle dependence of transactivation of gene expression, we isolated stable CHO cell lines that were transfected with the c-Myc reporter plasmid pMyc1E1bLuc. Synchronous populations of cells were prepared by using the release from hydroxyurea-induced growth arrest at the G₁/S phase boundary. The cells were then harvested at defined times as they traversed the cell cycle, and the luciferase activity present in the cell extracts was measured. It was observed that the luciferase activity exhibited a marked cell cycle dependence (Fig. 2A). The luciferase expression by G₁/S-arrested cells (zero hours following hydroxyurea withdrawal) was comparable to the activity of asynchronously growing cells. No marked change in luciferase activity was noted as the cells proceeded from G₁/S through S phase (0 to 5 h following hydroxyurea withdrawal). However, exit from S phase (Fig. 1 and 2C) coincided with an increase in luciferase activity. During G₂ the luciferase activity continued to increase until a maximal level was reached at or near the G₂/M boundary (9 h following hydroxyurea withdrawal). Mitosis was monitored by microscopic analysis of cell morphology (data not shown) and by analysis of cellular DNA content to observe the appearance of G₁ cells (Fig. 1). It was found that a marked decline in luciferase activity occurred as the cells progressed from the G₂/M to the G₁ phase of the cell cycle (Fig. 2A). A lower level of luciferase activity persisted during G₁. However, the luciferase activity then decreased at the G₁/S phase boundary (18 to 20 h following hydroxyurea withdrawal) coincident with increased [³H]thymidine incorporation (Fig. 2C). Significantly, a similar pattern of oscillating luciferase activity was noted as the cells passed through a second consecutive cell cycle (Fig. 2). However, the fold changes in activity were reduced compared with those in the first cycle, probably because of a decrease in cell synchrony. Similar changes in luciferase activity were observed when the cells were synchronized with aphidicolin or Hoechst 33372 (data not shown). Together, these data demonstrate that the luciferase expression by the c-Myc reporter plasmid pMyc1E1bLuc is cell cycle dependent with a marked increase during the S-to-G₂/M transition of the cell cycle.

Increased function of the c-Myc transactivation domain during the S-to-G₂/M transition of the cell cycle. The mechanistic basis for the cell cycle-dependent expression of the luciferase gene in cells transfected with the c-Myc reporter plasmid is unclear (Fig. 2A). Previous studies have established that c-Myc, Max, and c-Myc-Max heterodimers are expressed constitutively throughout the cell cycle (16, 28, 49, 61, 94, 112, 116). Therefore, the observed cell cycle-dependent regulation (Fig. 2A) is not caused by changes in the level of expression of c-Myc-Max complexes (16, 61). However, several other regulatory mechanisms could account for the cell cycle-dependent transactivation potential of c-Myc. As an initial approach to resolve these possible mechanisms, we investigated whether alterations in the

FIG. 2. Cell cycle-dependent transactivation of gene expression. CHO cells were released from hydroxyurea-induced growth arrest at the G₁/S boundary and then harvested at defined times. Luciferase activities in cell extracts were measured as light units per milligram of protein and are presented as normalized luciferase activities. (A) Luciferase activity was measured in extracts prepared from cells stably transfected with a reporter plasmid containing a c-Myc binding site (pMyc1E1bLuc). (B) Luciferase activity was measured in extracts prepared from cells stably cotransfected with the GAL4/Myc activator plasmid (pGAL4/Myc) and the GAL4 reporter plasmid (pG5E1bLuc). (C) [³H]thymidine incorporation into DNA during a 30-min period was measured. Similar data were obtained in three separate experiments.

function of the c-Myc transactivation domain could account for the cell cycle-dependent regulation of the reporter gene expression. As the c-Myc protein is composed of several functional domains, the experimental strategy that we employed was to construct a fusion protein consisting of the NH₂-terminal transactivation domain of c-Myc and the DNA binding domain of the yeast transcription factor GAL4 (59). This approach allows the direct analysis of the function of the c-Myc transactivation domain during the cell cycle.

We isolated stable CHO cells that expressed the GAL4/Myc fusion protein together with a luciferase reporter plasmid (pG5E1bLuc) that contains sites for GAL4 binding (Fig. 2B). The cells were synchronized by using release from hydroxyurea-induced growth arrest, and extracts were prepared at different times during the subsequent cell cycle. Western blot analysis demonstrated that the level of expression of the GAL4/Myc fusion protein was not markedly altered as the cells progressed through the cell cycle (Fig. 3). In some experiments a small decrease in GAL4/Myc expression was noted at 9 to 10 h following hydroxyurea withdrawal, but this decrease was not reproducibly observed (Fig. 3). Control experiments using the GAL4 fusion protein expression vector (pSG424) confirmed the conclusion that the expression of the GAL4/Myc fusion protein was not cell cycle dependent (Fig. 4C). In contrast to the constitutive expression of GAL4/Myc (Fig. 3), the transactivation of luciferase expression by GAL4/Myc was observed to be cell cycle dependent (Fig. 2B). A marked increase in the transactivation of luciferase reporter gene expression by the GAL4/Myc fusion protein was observed as the cells progressed from the S to the G₂/M phase of the cell cycle (6 to 9 h following hydroxyurea withdrawal). A second smaller increase in luciferase activity was noted during the G₁ phase of the cell cycle (10 to 18 h following hydroxyurea withdrawal). Similar cell cycle-dependent changes in luciferase activ-

ity were noted when the cells were synchronized with aphidicolin or with Hoechst 33372 (data not shown).

Control experiments demonstrated that the GAL4 reporter plasmid (pG5E1bLuc) exhibited no cell cycle-regulated expression in the absence of the GAL4/Myc fusion protein (Fig. 4B). Therefore, we conclude that the transac-

FIG. 3. Analysis of GAL4/Myc fusion protein expression during the cell cycle. Stable CHO cell transformants that express the GAL4/Myc fusion protein were released from hydroxyurea-induced growth arrest at the G₁/S boundary and then harvested at defined times. Asynchronously (AS) growing cells were also harvested for analysis of GAL4/Myc fusion protein expression. Nuclear extracts were prepared, and the GAL4/Myc proteins were isolated by immunoprecipitation with an anti-GAL4 antibody. The level of the GAL4/Myc fusion protein was examined by Western blot analysis using an anti-Myc antibody and enhanced chemiluminescence detection system. Similar results were obtained in three separate experiments.

FIG. 4. A point mutation at the c-Myc phosphorylation site Ser-62 causes decreased cell cycle-dependent transactivation of reporter gene expression. Luciferase activity was measured in extracts prepared from stably transfected CHO cells at different times following release from hydroxyurea-induced growth arrest. The luciferase activities were measured as light units per milligram of protein and are presented as normalized luciferase activities. (A) Transactivation of luciferase expression by GAL4/Myc (○) and GAL4/[Ala⁶²]Myc (●) was examined by using the GAL4 reporter plasmid pG5E1bLuc. (B) Basal activity of the GAL4 reporter plasmid (pG5E1bLuc) in the absence of the GAL4/Myc fusion protein. (C) The expression by the GAL4 fusion protein vector (pSG424) was examined by using the plasmid pSG424-Luc. Similar data were obtained in three separate experiments.

tivation potential of the GAL4/Myc fusion protein is regulated during the cell cycle. Significantly, the oscillation of transactivation potential observed for the GAL4/Myc fusion protein (Fig. 2B) was similar to cell cycle-dependent changes in luciferase activity detected in cells transfected with the c-Myc reporter plasmid (Fig. 2A). Together, these data

suggest that the regulation of the c-Myc reporter plasmid can be accounted for by cell cycle-dependent changes in the function of the c-Myc transactivation domain. Furthermore, these data establish that the observed cell cycle regulation of transactivation potential of c-Myc is independent of changes in the function of Max, Mad, and Mxi1.

FIG. 5. Increased phosphorylation of the c-Myc transactivation domain at the G₂/M phase of the cell cycle. COS-7 cells were transfected with 10 μg of the GAL4/Myc expression plasmid (pGAL4/Myc or pGAL4/[Ala⁶²]Myc). The cells were released from hydroxyurea-induced growth arrest for 2 h (S phase) or for 8 h (G₂/M phase). (A) Cells were labeled by incubation at 37°C for 4 h with 2 mCi of [³²P]phosphate per ml. The GAL4/Myc fusion proteins were immunoprecipitated with a rabbit anti-GAL4 antibody and analyzed by SDS-PAGE and autoradiography. (B) Immunoblot analysis of the cell lysates was used to measure the level of expression of the GAL4/Myc fusion proteins. The blot was probed with an anti-GAL4 antibody, and the Myc fusion proteins were visualized with ¹²⁵I-protein A and autoradiography. (C) The radioactivity was quantitated with a Phosphorimager and ImageQuant software (Molecular Dynamics Inc.) and the $^{32}\text{P}/^{125}\text{I}$ ratio for the GAL4/Myc fusion proteins was calculated. Normalized values for the $^{32}\text{P}/^{125}\text{I}$ ratio are presented. Similar results were obtained in three separate experiments.

A point mutation at the phosphorylation site Ser-62 suppresses the cell cycle-dependent regulation of the c-Myc transactivation domain. In previous studies it has been established that phosphorylation represents one mechanism of regulation of the c-Myc transactivation domain (45, 54, 55, 99, 100) and that the c-Myc protein is hyperphosphorylated at the G₂/M phase of the cell cycle (71). We therefore tested the hypothesis that phosphorylation is a mechanism that can account for the cell cycle regulation of the c-Myc transactivation domain. The phosphorylation state of the GAL4/Myc fusion protein was examined at the two stages of the cell cycle where the highest (G₂/M phase) and lowest (S phase) levels of transactivation potential were observed (Fig. 2B). In experiments using synchronized COS-7 cells, it was found that there was a marked increase in both the transactivation potential (data not shown) and the state of phosphorylation of the GAL4/Myc fusion protein at G₂/M compared with that at S phase (Fig. 5). Phosphoamino acid analysis revealed an

FIG. 6. Characterization of the cell cycle-dependent phosphorylation of the c-Myc transactivation domain by phosphoamino acid analysis and phosphopeptide mapping. The GAL4/Myc fusion proteins (Fig. 5) were analyzed by [³²P]phosphoamino acid analysis (panel A) and by tryptic [³²P]phosphopeptide mapping (panel B). The origin is marked with a cross at the lower left corner of each peptide map, and the electrophoretic (cathode at right) and chromatographic dimensions are illustrated. The exposure times for autoradiography were 5 days (panel A) and 7 days (panel B) at -80°C using Kodak X-OMAT AR film and a Dupont Lightning Plus enhancing screen.

increase in [³²P]phosphoserine and a small increase in [³²P]phosphothreonine (Fig. 6A). Tryptic phosphopeptide mapping demonstrated that the increased phosphorylation can be accounted for by a change in the level of a tryptic [³²P]phosphopeptide doublet (Fig. 6B). This phosphopeptide doublet has previously been demonstrated to be due to the presence of both limit and partial tryptic peptides corresponding to the region surrounding Ser-62 (2). Significantly, the replacement of Ser-62 with Ala resulted in the loss of this tryptic phosphopeptide doublet (Fig. 6B). Three additional minor tryptic [³²P]phosphopeptides were observed to be increased in maps obtained from cells at G₂/M compared with cells in S phase (Fig. 6B). Together, these data reveal a complex pattern of cell cycle-dependent phosphorylation of the c-Myc transactivation domain that includes (but is not limited to) phosphorylation at Ser-62.

To examine the role of phosphorylation of the c-Myc transactivation domain at Ser-62, the effect of mutation at this site was investigated. We isolated CHO cells that stably expressed the GAL4/[Ala⁶²]Myc fusion protein together with the pG5E1bLuc reporter plasmid. The cells were then

synchronized, and extracts were prepared for the analysis of luciferase activity as the cells passed through the cell cycle. It was observed that the mutation at Ser-62 caused a marked decrease in phosphorylation at the G₂/M phase of the cell cycle (Fig. 5 and 6). The mutation at Ser-62 also suppressed (but did not eliminate) the increased transactivation potential observed during the S-to-G₂/M transition of the cell cycle (Fig. 4A). Together, these data indicate a role for the phosphorylation of the c-Myc transactivation domain during cell cycle regulation of c-Myc function.

DISCUSSION

c-Myc function is thought to be required for cell cycle progression and cellular proliferation. As c-Myc expression is constitutive, it is possible that c-Myc may function throughout the cell cycle. Alternatively, it is possible that the critical functions of c-Myc are restricted to specific points within the cell cycle. As an initial approach to resolve this question, we have examined the cell cycle dependence of the transactivation potential of c-Myc.

Cell cycle-regulated expression of a reporter gene is conferred by the c-Myc DNA binding site CACGTG. We examined the cell cycle regulation of luciferase expression by using a synchronized population of CHO cells transfected with the c-Myc reporter plasmid pMyc1E1bLuc. It was observed that there was no marked change in reporter gene expression as the cells progressed through S phase. However, a pronounced increase in luciferase activity was noted during the S-to-G₂/M transition of the cell cycle (Fig. 2). A marked decrease in reporter luciferase expression coincident with mitosis was observed, as indicated by a decrease in cellular DNA content and the emergence of cells at the G₁ phase of the cell cycle. Following this, the reporter luciferase activity remained moderately high during G₁ until the G₁/S boundary, when a decline in activity was observed. The luciferase expression by CHO cells transfected with the c-Myc reporter plasmid therefore exhibits biphasic cell cycle regulation with a small increase during G₁ and a marked increase during G₂ (Fig. 2A).

We tested the hypothesis that the activity of the c-Myc transactivation domain contributes to the cell cycle regulation of the expression of luciferase activity observed in experiments using CHO cells transfected with the c-Myc reporter plasmid pMyc1E1bLuc (Fig. 2). The experimental approach that we employed was to construct a hybrid transcription factor consisting of the transactivation domain of c-Myc fused to the DNA binding domain of the yeast transcription factor GAL4 (59). Using this fusion protein strategy, we determined that the transactivation of gene expression mediated by this domain of c-Myc is cell cycle regulated with a marked increase in activity during the S-to-G₂/M transition. Thus, changes in the function of the c-Myc transactivation domain can account, in part, for the cell cycle-dependent regulation of the c-Myc reporter plasmid (Fig. 2).

Cell cycle-dependent phosphorylation of the c-Myc transactivation domain at Ser-62. Protein phosphorylation offers many useful features as a regulatory mechanism that permits the regulation of transcription factors and thereby gene expression (54, 55). Indeed, protein phosphorylation represents an important mechanism of regulation of the mammalian cell cycle (77). Recently, Lüscher and Eisenman (71) have presented evidence demonstrating that the phosphorylation state of c-Myc is regulated during the cell cycle, although the site(s) and functional significance of the phos-

phorylation were not defined. Here we report that Ser-62 is a site of cell cycle-regulated phosphorylation of c-Myc. This phosphorylation site is located within the NH₂-terminal transactivation domain of c-Myc (62) and is conserved in all members of the Myc family (2, 100).

Ser-62 is the major site of phosphorylation of c-Myc by MAP kinase in vitro (2) and it is possible that MAP kinase may phosphorylate c-Myc at this site in vivo (100). Recently, the molecular cloning of three rat MAP kinase isoforms (also referred to as extracellular signal-regulated kinases) has been reported by Boulton et al. (17, 18). A consensus sequence for the phosphorylation of substrates has been defined (2, 19, 41) and several protein substrates have been described: c-Myc (2); c-Jun (2, 93); p62^{TCF} (40); EGF receptor (25, 78, 109); cPLA₂ (69); p90^{sk} (108); and MAP-KAP kinase 2 (106). MAP kinases have been shown to be activated by treatment of cells with growth factors and other extracellular stimuli (20). In addition, MAP kinase activity is regulated during the cell cycle. A marked increase in MAP kinase activity is observed during the M phase of the meiotic cycle of oocytes derived from clams (102), sea stars (98), and *Xenopus laevis* (9, 38, 43, 44, 86, 87). MAP kinase has also been reported to be activated during the M phase of the mitotic cell cycle of *X. laevis* (44). In mammalian cells, the control of MAP kinase activity during the cell cycle has been less well defined. However, it has recently been demonstrated that MAP kinase is activated biphasically during the cell cycle of CHO cells with a small increase during G₁, a larger increase at G₂, and no activity during M phase (110). Significantly, this pattern of MAP kinase activation in CHO cells is similar to the observed cell cycle regulation of the c-Myc transactivation domain (Fig. 2A). Thus, MAP kinase could account for the cell cycle regulation of c-Myc phosphorylation in vivo. Glycogen synthase kinase-3 may also contribute to the phosphorylation of c-Myc at Ser-62 (97). However, as Ser-62 is a substrate for the cell cycle-regulated p34^{cdc2} protein kinase (99), it is likely that there is a prominent role for one or more cyclin-dependent protein kinases in the control of c-Myc phosphorylation. In addition, it is possible that there may also be a role for changes in protein phosphatase activity. Thus, the regulation of c-Myc phosphorylation at Ser-62 is likely to be complex and probably involves more than one kinase or phosphatase acting at different stages of the cell cycle.

Role of cell cycle-regulated phosphorylation of the c-Myc transactivation domain at Ser-62. To examine the significance of the cell cycle-dependent phosphorylation of the c-Myc transactivation domain, we investigated the effect of the replacement of Ser-62 with Ala. This mutation suppressed the cell cycle regulation of both the activity and phosphorylation state of the c-Myc transactivation domain (Fig. 4 and 5). The inhibited activity of the mutated Ala-62 c-Myc transactivation domain could be mediated by nonspecific structural alterations that may compromise the function of the transactivation domain under both basal and activated conditions. However, we have previously demonstrated that phosphorylation at Ser-62 positively regulates the activity of the c-Myc transactivation domain (45, 99, 100). Together, these considerations indicate that the phosphorylation site Ser-62 accounts, in part, for the cell cycle regulation of c-Myc function.

Although the replacement of Ser-62 with Ala greatly diminished the increase in transactivation observed during the G₂ phase of the cell cycle, this mutation did not completely block the cell cycle regulation of reporter gene expression (Fig. 4). This result indicates that mechanisms in

addition to phosphorylation at Ser-62 must also contribute to the cell cycle regulation of c-Myc function. Phosphopeptide mapping demonstrates that while Ser-62 represents a major site of cell cycle-regulated phosphorylation, additional sites of c-Myc phosphorylation also exist (Fig. 6). It is therefore possible that the phosphorylation of c-Myc at one or more of these additional sites may also contribute to the cell cycle regulation of c-Myc activity. A regulatory mechanism that is independent of c-Myc phosphorylation could also contribute to the changes in transactivation potential observed during the cell cycle. One plausible mechanism is the regulation of Max (10), although this would not account for cell cycle-dependent transactivation caused by the GAL4/Myc fusion protein (Fig. 2). Alternatively, it is possible that an interaction of the c-Myc transactivation domain with a heterologous protein is required for cell cycle regulation. Indeed, c-Myc is known to bind to the nuclear matrix (36, 115). Furthermore, *in vitro* studies have shown that the c-Myc transactivation domain binds to the retinoblastoma protein, Rb (96). It is therefore possible that an Rb-like protein could contribute to the cell cycle regulation of c-Myc function *in vivo* (29, 57, 117).

Role of c-Myc in the S-to-G₂/M transition of the cell cycle. It is known that nuclear proto-oncogenes can modulate the expression of critical genes involved in cell cycle progression (47, 53). Although there is strong evidence to suggest a role for c-Myc in the regulation of the cell cycle, the molecular mechanism of c-Myc action and the stage(s) of the cell cycle influenced by c-Myc are not well understood. In previous studies, a role for c-Myc during G₁ or at the G₁/S boundary has been indicated (6, 34, 35, 42, 50, 51, 58, 65, 83, 84). The observation of elevated transactivation of the c-Myc reporter plasmid during G₁ (Fig. 2) is consistent with the hypothesis that c-Myc functions during the G₁ phase of the cell cycle. However, the major peak of transactivation of the c-Myc reporter plasmid was found during the S-to-G₂/M transition (Fig. 2) indicating that c-Myc may also have an important function during the G₂ phase of the cell cycle.

One of the functions of G₂ is to ensure that S phase ends before cell division begins, thereby guaranteeing an obligatory temporal relationship between DNA replication and mitosis (77). On the basis of this information, we propose a role for c-Myc in the regulation of the expression of one or more genes that are required for the cell to progress from S phase to mitosis. Evidence that supports this hypothesis has recently been reported by Shibuya et al. (103) in experiments designed to characterize a defect in the EGF-stimulated growth of BAF-BO3 cells. Treatment of these cells with EGF results in entry into S phase, but further progression through the cell cycle is blocked. Significantly, this defect can be corrected by the ectopic expression of the *c-myc* gene, indicating that c-Myc is required for progression through G₂ (103). These observations, together with the results of the present study, strongly support the contention that c-Myc may have an important function during the G₂ phase of the cell cycle.

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