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Differential modulation of cyclin gene expression by *MYC*

(G_1 phase cyclins/cell cycle)

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ABSTRACT **We have investigated the effects of deregulated expression of the human c-*MYC* protooncogene on cyclin gene expression and on the transcription factor E2F. We found that constitutive expression of *MYC* or activation of conditional MycER chimeras led to higher levels of cyclin A and cyclin E mRNA. Activation of cyclin A expression by *MYC* led to a growth factor-independent association of cyclin A and cdk2 with the transcription factor E2F and correlated with an increase in E2F transcriptional activity. In contrast, expression of the G_1 phase cyclin D1 was strongly reduced in *MYC*-transformed cells. In synchronized cells, repression of cyclin D1 by *MYC* occurred very early in the G_1 phase of the cell cycle.**

The mechanism by which deregulated expression of the c-*MYC* protooncogene transforms cells and interferes with cellular growth control has been the subject of intensive investigation during the last years (for a recent review, see ref. 1). The c-*MYC* gene is part of a small gene family that encodes nuclear phosphoproteins with extremely short half-lifes (2). The c-Myc protein has the capacity to bind to DNA in a sequence-specific manner and recognizes an "E-box" motif with the central sequence CACGTG (3, 4). Its affinity to DNA is greatly enhanced by heterodimerization with a second protein, termed Max, that is structurally closely related to Myc (5, 6). In transient transfection assays, expression of *MYC* appears to stimulate expression of promoters that contain the E-box motif, whereas expression of *MAX* is inhibitory (7, 8).

The c-Myc protein appears to be a key regulator of cell proliferation. This view is supported mainly by two lines of evidence. (*i*) The expression of c-*MYC* closely correlates with cell proliferation *in vivo* during embryogenesis (9) and in various experimental systems (10). (*ii*) Deregulated expression of c-*MYC* leads to an almost complete loss of cell cycle control in response to external factors (11). For example, we have shown that activation of conditional alleles of c-*MYC* (MycER chimeras, see below) is sufficient to stimulate growth factor-deprived cells to enter into and progress through the cell cycle (12). How the c-Myc protein exerts these effects is unknown. One hypothesis is that c-*MYC* controls the expression of genes that are important for cell cycle progression. We have previously described one gene, α-prothymosin, that appears to be directly regulated by c-*MYC* *in vivo* (12). However, the function of α-prothymosin is a conundrum. Recently, from a number of experimental approaches, candidate genes that may control cell cycle progression have been isolated: these genes include cyclins, cyclin-dependent kinases, and the products of two tumor-suppressor genes, p53 and the retinoblastoma (Rb) gene (for a recent review, see ref. 13). Whether *MYC* directly controls expression of any of these genes is unknown.

Myc might also form direct protein–protein complexes with cell cycle regulators, such as the Rb protein, thereby modulating their function. Support for this hypothesis stems from the functional (14) and limited structural homology (15) between Myc and the adenovirus protein E1A. E1A physically associates with the Rb protein (16) and dissociates it from its association with a cell cycle-regulated transcription factor, E2F (17, 18). Indeed, a protein complex between Myc and the Rb protein has recently been demonstrated *in vitro*; whether it also exists *in vivo* is unknown (19).

To gain insight into the mechanism(s) by which c-*MYC* affects cellular proliferation, we have investigated how constitutive expression of c-*MYC* or activation of MycER chimeras affects cyclin gene expression and a cyclin-dependent transcription factor, E2F. We find that *MYC* induces cyclin A and cyclin E expression and leads to a growth factor-independent association of cyclin A with E2F. In contrast, very early in the cell cycle, *MYC* acts to suppress expression of cyclin D1.

MATERIALS AND METHODS

Cell Lines. BALB/c-3T3 mouse fibroblasts were infected with recombinant retroviruses expressing the human *MYC* gene under the control of the murine sarcoma virus (MSV) 5′ long terminal repeat or a control retrovirus (12). After selection with neomycin, resistant colonies were pooled and analyzed. All other manipulations of these cells were carried out as described (12). Cell cycle analysis was performed using a FACScan instrument after staining cells with propidium iodide (10 μg/ml). Nocodazole was used at a final concentration of 40 ng/ml.

Northern Blots. Total RNA was extracted by the guanidinium/acid phenol method (20). *MYC* expression was analyzed with a 1.4-kb human cDNA fragment (21). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was analyzed with a rat cDNA probe (22). Expression of cyclin A was analyzed with a 1.6-kb mouse probe (J. Wessbecher), cyclin E was analyzed with a 1.6-kb mouse probe (P.S., unpublished data; ref. 23), and cyclins D1 and D2 was analyzed with the mouse cyl1 and 2 probes (24). Quantitation of autoradiograms was carried out with a densitometer.

Stable Transfections and Reporter Gene Assays. A clone of RAT1A-MycER cells (21) was cotransfected with 2 μg of pSV2-hygro and 8 μg of either EII-lac or a mutant derivative of EII-lac deleted for both E2F sites (ΔEII-lac) (25). Two days after transfection, hygromycin was added to a final concentration of 200 μg/ml. Resistant colonies were pooled after 10 days of selection. All other manipulations with these

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Abbreviations: Rb, retinoblastoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CS, newborn calf serum; MSV, murine sarcoma virus.

cells were as described (12). Whole cell extracts were prepared by a freeze–thaw method and β-galactosidase assays were carried out with *o*-nitrophenyl β-D-galactoside.

Bandshift Assays and Western Blots. Whole cell extracts were prepared from cells as described (26). Six micrograms (or 12 μg where indicated) of either extract was incubated with a 5′ end-labeled DNA fragment corresponding to an E2F binding site from the adenovirus E2 promoter (27) or, as a control, to an E-box sequence (28). Immunoshift assays and Western blots were performed as described (26).

RESULTS

Stimulation of Cyclins E and A by *MYC*. To investigate the effects of c-*MYC* on the expression of cell cycle regulatory genes, we infected murine BALB/c-3T3 cells with a recombinant retrovirus expressing the wild-type human c-*MYC* gene under control of the MSV long terminal repeat (MSV-MYC). BALB/c-3T3 cells were also infected with the appropriate control retrovirus. Stable cell lines were derived in both cases using neomycin selection. Resistant colonies were pooled; expression of the exogenous human *MYC* gene was documented by Northern and Western blots (not shown). RNA was prepared from both cell lines under growing and serum-starved conditions and probed for RNA levels of cyclins A and E (Fig. 1) and of cdk2 (data not shown). For this analysis, cyclin A and cyclin E cDNAs were isolated from a mouse cDNA library and partially sequenced (not shown). Under conditions of serum starvation, expression of *MYC* significantly induces cyclin E and cyclin A. No significant difference was induced by *MYC* in exponentially growing cells. The extent of *MYC*-dependent stimulation differed significantly between the two genes: whereas cyclin A expression was stimulated 10-fold by *MYC* in starved cells, cyclin E expression was stimulated 3-fold. Expression of cdk2 was not affected by *MYC*.

To confirm that *MYC* can induce expression of cyclins A and E we made use of a previously established cell line, RAT1A-MycER, producing conditionally active Myc protein (21). This cell line had been obtained by infecting RAT1A cells with a recombinant retrovirus encoding a chimeric protein consisting of human c-Myc and the hormone binding domain of the human estrogen receptor. It displays an estrogen-dependent, conditionally transformed phenotype (21). Activation of MycER in confluent cells leads to partial reentry into the cell cycle (12); under these conditions, changes in cyclin A and E mRNA levels were analyzed. The results are presented in Fig. 2. They confirm that activation of MycER is sufficient to stimulate expression of cyclin E and cyclin A. As in BALB/c-3T3 cells, the extent of induction differed significantly: cyclin A mRNA levels increased 8-fold after addition of estrogen; a similar stimulation was observed for α-prothymosin (12). In contrast, cyclin E was only stimulated about 3-fold relative to GAPDH. Interestingly, cyclin A and E expression were activated with similar kinetics by *MYC*.

Activation of Cyclin A Expression Correlates with Increased Transcriptional Activity of E2F. To determine the functional consequences of cyclin A activation, we analyzed the effect of *MYC* on the composition and the activity of the transcription factor E2F. Cyclin A is associated with E2F during S phase (29); in untransformed cells, E2F is also complexed with the Rb gene product (pRb) during the G_1 phase, an interaction that leads to repression of E2F activity (17, 30, 31). Furthermore, E2F has been shown to bind to p107 (32), a protein related to pRb (33).

This analysis was initially carried out by comparing the E2F composition between control and *MYC*-expressing BALB/c-3T3 cells under growing and serum-starved conditions; to determine the functional consequences of the differences we observed, it was later repeated in RAT1A-MycER cells. The effect of *MYC* was identical in both

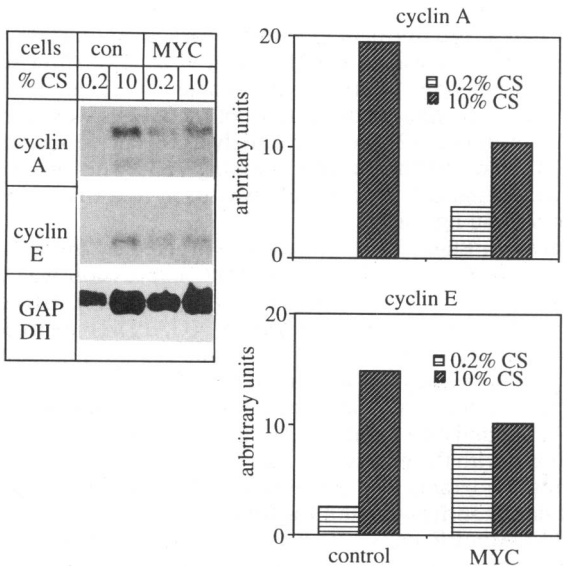

FIG. 1. Expression of *MYC* causes serum-independent expression of cyclin A and cyclin E. Northern blot analysis of RNA from either control (con) or *MYC*-expressing fibroblasts grown in 10% serum or shifted to 0.2% serum for 48 hr. (*Left*) Expression of cyclin A, cyclin E, and GAPDH. CS, newborn calf serum. (*Right*) Quantitative representation of the results. Northern blots for cyclin A show two bands in mouse and rat fibroblasts; the exact nature of each band is unknown.

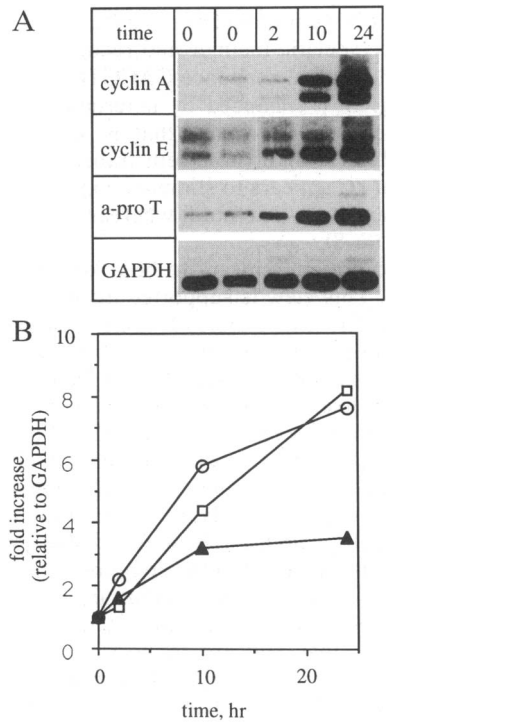

FIG. 2. Activation of cyclins E and A by activation of MycER chimeras. (*A*) RAT1A-MycER cells were grown to confluence before estrogen was added to a final concentration of 200 nM. At the indicated times, RNA was prepared and the amount of cyclin A, cyclin E, α-prothymosin (a-pro T), and GAPDH mRNA was determined. Northern blots for cyclin E show two bands in RAT1A cells. (*B*) Graphic representation of the results. ○, Cyclin A; ▲, cyclin E; □, α-prothymosin.

experimental systems. The results are shown for RAT1A-MycER cells in Fig. 3.

In RAT1A-MycER cells grown to confluence in the absence of estrogen we detected two major E2F complexes, one of which contains the Rb protein in addition to the transcription factor. The protein composition of the second complex, designated X in Fig. 3*A*, is not known. Furthermore, we observed an additional very weak complex containing cyclin A and cdk2 and some additional complexes of unknown protein composition (Fig. 3*A*).

E2F complexes present after activation of *MYC* differed in two respects from those described above. (*i*) Using the same amount of cellular protein, E2F binding activity was significantly higher in cells treated with estrogen for 20 hr. (*ii*) We observed an additional, slower migrating complex that contains E2F complexed with cyclin A and cdk2, as judged from titration by both antibodies (Fig. 3*A*). Antibodies raised against the Myc protein and the estrogen receptor, respectively, did not interfere with the bandshift pattern in both conditions.

The amount of E2F complexed with pRb was the same before and after activation of *MYC*. Thus, complexes between E2F and the Rb protein appeared not to be significantly affected by the Myc protein (see below); time course experiments (not shown) confirmed that no transient change in E2F composition had been missed in these experiments. In a control experiment the same extracts were tested for binding to the E-box sequence CACGTG, which has been shown to bind several proteins, among which the upstream stimulating factor (USF) is predominant in these cells (A. Schneider, unpublished results). Under these conditions, binding of the Myc protein to E-box sequences is not detectable. Binding to this sequence was not altered significantly by activation of MycER (not shown), indicating that the changes we observe on E2F are specific for this transcription factor.

In BALB/c-3T3 cells, expression of *MYC* did not induce significant changes in E2F composition if cells were grown in high serum concentration; however, in cells that express *MYC*, cyclin A and cdk2 associate with E2F even under conditions of serum starvation. As seen previously in RAT1A-MycER cells, constitutive expression of *MYC* did not affect the amount of E2F/Rb complexes seen in these cells (not shown).

From these results we conclude that an increase in the amount of the E2F/cyclin A/cdk2 complex represents the major change imposed on this transcription factor by the activation of *MYC*. In contrast to the nuclear oncogenes of DNA tumor viruses (18, 34), Myc appears not to dissociate E2F/Rb complexes.

To assess the functional consequences of the observed changes, a clone of RAT1A-MycER cells was stably transfected with a reporter construct containing the bacterial β-galactosidase gene driven by the adenovirus E2 promoter, containing two E2F binding sites. To assess the contribution of E2F to β-galactosidase transcription in this system, RAT1A-MycER cells were also transfected by the construct E2 β-gal ΔE2F, which is identical to the wild-type construct except that both E2F binding sites are mutated (25). Both constructs were cotransfected with a plasmid providing resistance to hygromycin. About 20–50 resistant colonies were observed in each transfection and pooled for subsequent experiments. To determine whether activation of *MYC* could

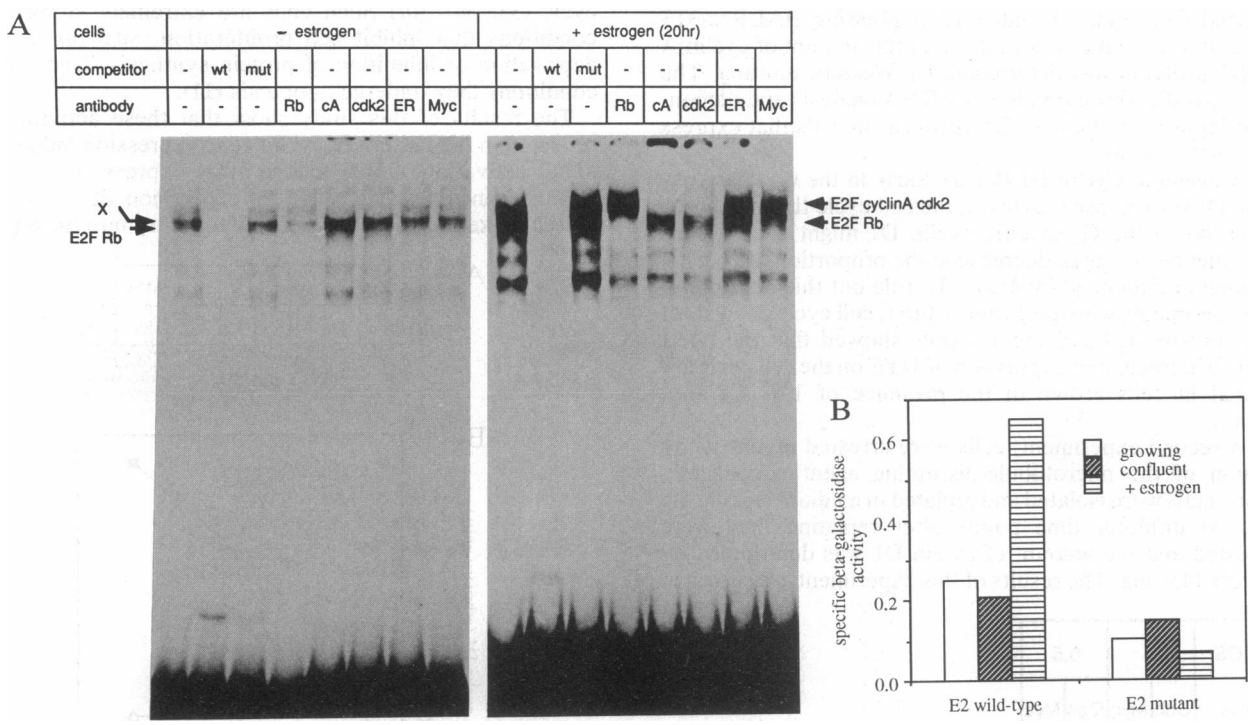

FIG. 3. Activation of *MYC* induces association of cyclin A and cdk2 with the E2F complex and increases β-galactosidase activity controlled by the adenovirus E2 promoter. (*A*) E2F bandshifts were performed with extracts from MycER cells in the absence (*Left*) and the presence (*Right*) of estrogen for 20 hr. Specificity of the complexes was demonstrated by competition with a 50-fold excess of a wild-type (wt) or mutated (mut) E2F binding site (27). Complexes were challenged by antibodies to pRb, cyclin A (cA; ref. 34), cdk2 (26, 53), the human estrogen receptor [ER (F3), kindly provided by P. Chambon, Centre National de la Recherche Scientifique, Strasbourg, France], and human Myc (monoclonal antibody 9E10) as indicated. "X" denotes a form of the E2F transcription factor that reacts with none of the antibodies used in this study; X migrates very close to E2F-Rb. However, addition of anti-Rb antibodies clearly reveals the existence of a second band in this part of the gel. (*B*) RAT1A-MycER cells were stably cotransfected either with an EII-lac construct or with a mutant derivative lacking both E2F (ΔEII-Lac) sites and a hygromycin-resistance plasmid (pSV2-hygro). Resistant colonies were pooled and specific β-galactosidase activities were determined in either confluent or exponentially growing cells before or 48 hr after addition of estrogen. Activities are expressed as OD_{420} milliunit/hr per μg of protein.

stimulate E2F activity, both cell lines were grown to confluence (to reduce levels of endogenous c-*MYC* expression as much as possible) in the absence of estrogen, before *MYC* was activated by the addition of estrogen. Extracts were prepared at different time points after addition of the hormone and assayed for β-galactosidase activity. The results of a representative experiment are shown in Fig. 3*B*. We observed a 3-fold stimulation of β-galactosidase activity in the cell line expressing the wild-type E2 promoter after addition of estrogen. This transcriptional stimulation of the E2 promoter depends entirely on intact E2F binding sites since under the same conditions the mutated construct is not activated; in this case, addition of estrogen led to a slight repression of β-galactosidase activity (Fig. 3*B*). Surprisingly, β-galactosidase activity could be stimulated by estrogen addition even in exponentially growing cells (Fig. 3*B* and data not shown), indicating that under the conditions of the experiment the retroviral MycER construct can stimulate E2F activity beyond the level effected by the endogenous c-*MYC* gene.

Effect of *MYC* Expression on D Cyclins. To determine how Myc affects expression of D cyclins, RNA was prepared from cells grown as described in the legend to Fig. 1 and hybridized to probes for cyclin D1 and D2 and, as a control, cyclin A. The results of this experiment are shown in Fig. 4. We observed that, in contrast to cyclins A and E, cyclin D1 is strongly suppressed by *MYC* in growing cells; indeed, growing cells that express *MYC* show levels of cyclin D1 mRNA similar to those of serum-starved control cells. In the same samples, cyclin D2 is virtually unaffected by Myc. Cyclin A expression is induced in a manner similar to that shown in the experiment of Fig. 1. To confirm these results, extracts were prepared from control and *MYC*-expressing BALB/c-3T3 cells under the same conditions, and the amount of cyclin A and D1 proteins was determined by Western blotting. The results parallel those obtained by RNA analysis and demonstrate repression of cyclin D1 synthesis in cells that express *MYC* (not shown).

Repression of Cyclin D1 Occurs Early in the G_1 Phase. As cyclin D1 is expressed earlier in the cell cycle than cyclin A, repression of the G_1-specific cyclin D1 might simply be a consequence of a large decrease in the proportion of G_1 cells that might be induced by *MYC*. To rule out this possibility, two experiments were performed. First, cell cycle analysis of *MYC*-transformed and control cells showed that the overt effects of deregulated expression of *MYC* on the cell cycle are minimal in cells grown in the presence of 10% CS (not shown).

In a second experiment, cells were arrested in mitosis by addition of the microtubule-disrupting agent nocodazole. Mitotic cells were isolated and replated in medium lacking the drug. At different time points after replating they were harvested and the amount of cyclin D1 was determined by Western blotting. The results of this experiment are shown in Fig. 5*A* together with a quantitative representation of the results in Fig. 5*B*. As a control, progress through the G_1 phase was monitored by FACScan analysis and by cyclin A immunofluorescence (not shown).

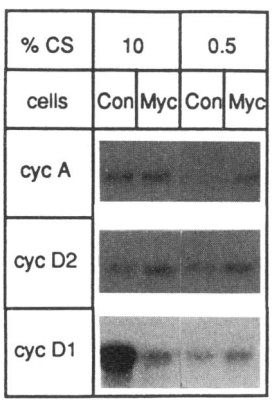

FIG. 4. Repression of cyclin D1 by *MYC*. Northern blot analysis of RNA from either control (Con) or *MYC*-expressing BALB/c-3T3 cells grown in 10% serum or shifted to 0.5% serum for 72 hr. The panels show expression of cyclins A, D1, and D2.

The amount of cyclin D1 increases early in the cell cycle of control cells. The strongest increase is seen between 3 and 6 hr after mitosis. Cells that express *MYC* have similar levels of cyclin D1 shortly after mitosis, but no increase in cyclin D1 expression is observed as cells progress through the G_1 phase of the cycle. *MYC*-expressing and control cells did indeed progress through the G_1 phase, as demonstrated by a large increase in the number of cells that were positive for cyclin A immunofluorescence (not shown).

These data rule out the possibility that repression of cyclin D1 is secondary to the effects of *MYC* on the cell cycle as it occurs under conditions where the cells are synchronized by the experimental regime. Second, they demonstrate that repression of cyclin D1 by *MYC* is due to failure to induce the gene in response to cell cycle progression. Third, they show that repression of cyclin D1 by *MYC* occurs very early in the cell cycle around a time where cells become committed for a new round of DNA replication.

DISCUSSION

Deregulated expression of *MYC* or activation of conditional alleles of Myc has several distinct effects on cell proliferation. (*i*) *MYC* exerts a strong mitogenic effect: for example, activation of MycER chimeras is sufficient to cause quiescent mouse fibroblasts to reenter the cell cycle in the absence of growth factors (12). (*ii*) Cells that express *MYC* constitutively show an accelerated passage through the G_1 phase of the cell cycle (35, 36). (*iii*) Such cells are extremely sensitive to conditions that inhibit cell proliferation, such as nutrient deprivation or inhibition of protein synthesis. Under these conditions they undergo apoptosis (11).

The results of this study show that these activities are reflected in the pattern of cyclin gene expression induced by *MYC*: activation of *MYC* can activate expression of cyclins E and A in quiescent cells. As expression of cyclin A is closely linked to and a prerequisite for entry into the S phase

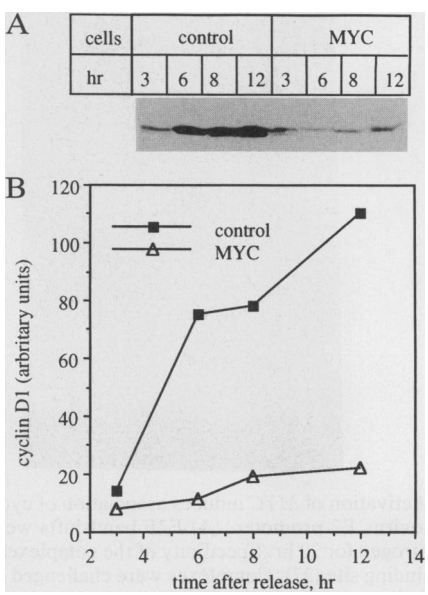

FIG. 5. Repression of cyclin D1 occurs early in the cell cycle. Cells were arrested in mitosis by treatment with nocodazole (40 ng/ml) for 18 hr. Mitotic cells were isolated and replated by incubating in drug-free medium. At the indicated times, samples were isolated and the amount of cyclin D1 (*A*) was determined. (*B*) Graphic representation of the results.

of the cell cycle (37), induction of this gene probably reflects the mitogenic potential of *MYC*.

In the presence of growth factors (10% CS), cells that express *MYC* have similar levels of cyclins A and E as control cells. However, they differ from their normal counterparts in expressing significantly lower levels of cyclin D1. Cyclin D1 is expressed earlier in the cell cycle than either cyclin E and A (24). Repression of cyclin D1 in our experimental system does not, however, merely reflect a decrease in the proportion of cells in the G_1 phase of the cell cycle: the experiment shown in Fig. 5 demonstrates that repression of cyclin D1 occurs even in synchronized cells and clearly rules out this possibility.

Activation of cyclin A expression by *MYC* leads to a growth factor-independent association of cyclin A with the transcription factor E2F. This is one of two changes that *MYC* induces in this transcription factor: time course experiments establish that entry of cyclin A along with cdk2 is preceded by an increase in total E2F binding activity. In our experiments these changes correlate with an increase in the transcriptional activity of E2F as measured on the adenovirus E2 promoter. Whether the increase in cyclin A levels causes the observed stimulation remains to be determined. Neither constitutive expression nor activation of conditional alleles of *MYC* appears to interfere with the association of the Rb protein with the E2F transcription factor. An association of Myc with pRb has been observed *in vitro* (19); our data suggest that if this interaction exists *in vivo*, its functional consequences appear to be different from the association between pRb and the nuclear oncogenes of DNA tumor viruses.

Cyclin D1 provides a new example of genes repressed by *MYC*. The Myc protein has been demonstrated to associate with a partner protein, Max, and bind to a specific DNA sequence with the core consensus CACGTG (3–6). Coexpression of Myc and Max stimulates expression from artificial promoters that contain such sequences (7, 8). However, enforced expression of *MYC* represses a number of genes, including collagen genes (38), the *neu* protooncogene (39), and, most notably, the c-*myc* gene itself (40). The mechanism of this repression has not been elucidated. Repression of cyclin D1 by *MYC* in our experimental system occurs as a failure to activate the gene in response to progression early in the cell cycle, as the basal level shortly after mitosis appears to be very similar between *MYC*-expressing and control cells (Fig. 5). Very low levels of cyclin D1 have also been observed in 293 cells that express the adenovirus E1A protein (M.P., J. Lukas, and G.D., unpublished results). Repression of cyclin D1 may, therefore, reflect a property that Myc shares with the adenovirus E1A protein.

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