MOLECULAR AND CELLULAR BIOLOGY, June 1994, p. 4032–4043
0270-7306/94/$04.00+0

Vol. 14, No. 6

# Repression of Cyclin D1: a Novel Function of MYC

ANGELIKA PHILIPP,[1] ANDREAS SCHNEIDER,[1] IMRE VÄSRIK,[2] KERSTIN FINKE,[1] YUE XIONG,[3] DAVID BEACH,[3] KARI ALITALO,[2] AND MARTIN EILERS[1]

*Zentrum für Molekularbiologie Heidelberg, 69120 Heidelberg, Federal Republic of Germany[1]; Molecular Cancer Biology Laboratory, Department of Pathology, University of Helsinki, 00014 Helsinki, Finland[2]; and Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724[3]*

Received 9 November 1993/Returned for modification 5 January 1994/Accepted 16 March 1994

**Constitutive expression of human *MYC* represses mRNA levels of cyclin D1 in proliferating BALB/c-3T3 fibroblasts. We expressed a series of mutant alleles of *MYC* and found that downregulation of cyclin D1 is distinct from previously described properties of MYC. In particular, we found that association with Max is not required for repression of cyclin D1 by MYC in vivo. Conversely, the integrity of a small amino-terminal region (amino acids 92 to 106) of MYC is critical for repression of cyclin D1 but dispensable for transformation of established RAT1A cells. Runoff transcription assays showed that repression occurs at the level of transcription initiation. We cloned the promoter of the gene for human cyclin D1 and found that it lacks a canonical TATA element. Transcription starts at an initiator element similar to that of the adenovirus major late promoter; this element can be directly bound by USF in vitro. Expression of *MYC* represses the cyclin D1 promoter via core promoter elements and antagonizes USF-mediated transactivation. Taken together, our data define a new pathway for gene regulation by MYC and show that the cyclin D1 gene is a target gene for repression by MYC.**

The proto-oncogene c-*myc* was identified as the cellular homolog of the transforming oncogene of chicken myelocytomatosis virus MC29 (16, 79). Expression of c-*myc* is tightly regulated and closely correlates with the proliferative status of a cell (19, 31, 39, 50, 80, 81). Deregulated expression of c-*myc* is often observed in tumor cells (26, 73). In both tissue culture and transgenic animals, introduction of a c-*myc* gene under the control of a strong constitutive promoter contributes to transformation and tumorigenesis; in some cells, it is sufficient for transformation (for a recent review, see reference 49).

Cells that carry a constitutively expressed c-*myc* gene lose the ability to control cell proliferation in response to growth factors; when deprived of such factors, they show few signs of cell cycle arrest but often undergo apoptosis (24, 38). This is illustrated by cells that express a chimeric protein containing the hormone-binding domain of the human estrogen receptor attached to the carboxy terminus of the human Myc protein. Transformation by these MycER chimeras is tightly dependent on the addition of estrogen (21). When exposed to estrogen, cells that carry such chimeras enter and progress through the cell cycle even in the absence of growth factors (22). At the same time, part of the cell population undergoes apoptosis (24). Also, constitutive expression of *MYC* leads, in some cells, to a characteristic shortening of the cell cycle which is not observed with other retroviral oncogenes (34, 65).

Control of cell proliferation may be one way by which *MYC* contributes to cell transformation; how *MYC* exerts this control is, however, unclear. The gene encodes a nuclear phosphoprotein (28, 56) that binds to specific DNA sequences (termed E boxes) (10, 60) and activates transcription upon binding to DNA (3, 35, 42). Several genes that carry E boxes have been suggested to be directly regulated by MYC (7, 8, 22, 27a, 78). Both in vitro and in vivo, the Myc protein heterodimerizes with a second helix-loop-helix protein called Max (11, 12, 59). Binding of Myc to Max is a prerequisite for both transcriptional activation (3, 5, 42) and transformation (2, 4).

More recently, Myc has been reported to heterodimerize in vitro with two proteins that recognize initiator (Inr) elements of transcription, a helix-loop-helix protein termed TfII-I (63, 64), and YY-1 (71). Heterodimerization with Myc precludes these proteins from activating transcription either in vitro (63) or in transient transfection assays (71), suggesting that MYC might repress genes via this pathway. Effects of MYC on cell proliferation and transformation might, therefore, result from either activation or repression of target genes; however, among the genes that are known to mediate progression through the cell cycle, those that are direct targets of MYC have not been identified.

Transitions in the mammalian cell cycle are thought to be mediated by complexes between a set of related kinases, termed cdk's (for cyclin-dependent kinase), and their regulatory subunits, termed cyclins (for a recent review, see reference 41). A number of cyclin genes have been identified that differ in the phase of the cell cycle in which they are expressed (for a recent review, see reference 70). Expression of cyclin genes is often regulated at the transcriptional level (43, 48, 51, 57). Generally, expression levels of different cyclins are high in proliferating cells (57, 82). However, individual cyclin genes differ in their expression patterns in response to nuclear oncogenes (14, 74), anti-oncogenes (30, 52), and conditions such as senescence (45), suggesting that they are not functionally equivalent. Indeed, biochemical differences between cyclins expressed early in the mammalian cell cycle have emerged (18, 25, 30, 36).

We have recently reported that constitutive expression of human *MYC* in BALB/c-3T3 cells results in elevated levels of both cyclin A and E mRNAs and repression of cyclin D1 mRNA (32). Similar results have been obtained with Rat6 cells (33) and rat embryo fibroblasts (44). Repression of cyclin D1 occurred under conditions in which expression of *MYC* induced no overt changes in cell cycle distribution. It occurred

* Corresponding author. Mailing address: Zentrum für Molekularbiologie Heidelberg (ZMBH), Im Neuenheimer Feld 282, 69120 Heidelberg, Federal Republic of Germany. Phone: 49-6221-566869. Fax: 49-6221-565894.

also in synchronized cells, demonstrating that it is independent of secondary effects of Myc on the cell cycle.

These data suggested that MYC has the ability to repress genes in addition to its transactivating functions. We show here that (i) repression of cyclin D1 by Myc does not require association with Max in vivo, (ii) it occurs on the level of transcription, (iii) it is mediated by core elements of the cyclin D1 promoter, and (iv) it antagonizes USF-mediated activation of the cyclin D1 promoter. Together, these data define a novel transcriptional property of the Myc protein.

## MATERIALS AND METHODS

**Deletion mutagenesis.** Deletions in the amino terminus of human *MYC* were introduced by PCR (29) into plasmid pSP65-cmyc (21). The first PCR was done with a primer spanning the start site of translation (5′-GGGATCCGAATTCACCATGCCCCTCAACGTT-3′) and a primer introducing the deletion. The sequences of these primers were 5′-CCCGGAGCGGCGGCTCGCCGGGGGCTGCAG-3′ for Δ45-63, 5′-CACCATGTCTGCTCGCCCGCCACCGCCGTC-3′ for Δ92-106, 5′-CTGGATGATGATGTTTCCTCCCAGCAGCTC-3′ for Δ109-126, and 5′-CTTCTCTGAGACGAGGTTTTTGATGAAGGT-3′ for Δ128-143. A second reaction was performed with primer 5′-GCCCGGGAACATCGATTTCTTCCTC-3′, which hybridizes to a region encompassing the *Cla*I site near the exon II-exon III boundary and primers starting at the deletion site; these were 5′-AGCCGCCGCTCCGGGCTC-3′ for Δ45-63, 5′-GGAGGAGACATGGTGAAC-3′ for Δ92-106, 5′-AACATCATCATCCAGGAC-3′ for Δ109-126, and 5′-CTCGTCTCAGAGAAGCTG-3′ for Δ128-143. The products of both reactions were mixed together, and a third reaction was carried out with the underlined primers. PCR products corresponding to the full-length product were excised, digested with *Eco*RI and *Cla*I, and cloned into plasmid pSP65-cmyc. This strategy introduces a Kozak consensus sequence at the translation start ATG codon.

The presence of the correct mutations was verified by sequence analysis (66). The sequence of In104 has not previously been reported; it is 5′-GTG ACC GAG CTC GAG CTC GAG CTG-3′, which encodes V T E L E L E L L G (the sequence of wild-type [wt] Myc is V T E L L G; the valine is amino acid 102). In vitro transcription was carried out with SP6 polymerase, and translation was done with a reticulocyte lysate in accordance with the manufacturer's (Promega) instructions. Subsequently, the mutated *MYC* genes were cloned into retroviral vector pMV7 (40).

For transient transfection assays, the *MYC* gene from pSP65-cmyc was inserted into the *Sma*I site of pUHD10-1, a cytomegalovirus (CMV)-based expression vector (17). CMV-Myn was a kind gift of G. Prendergast and has been described previously (58). For reporter plasmids, fragments of the cyclin D1 promoter were inserted into luciferase vector pXP-1 (53).

**Cell culture.** Cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (GP+E86 and RAT1A cells) or 10% newborn calf serum (BALB/c-3T3 cells) at 37°C in a 5% $CO_2$ atmosphere.

DNA was introduced into packaging cell line GP+E86 (47) by calcium phosphate precipitation (66). Selection of transfected cells was performed for 10 days by adding 800 μg of G418 per ml to the culture medium. Subsequently, cells were pooled and the virus was harvested from the cell supernatant (13). For infection, $2 \times 10^5$ cells (BALB/c-3T3 or RAT1A) were incubated with virus-containing supernatant and 8 μg of Polybrene per ml for 4 h. Selection for infected cells was carried out as described above; G418-resistant colonies were pooled for subsequent experiments. Colony formation in soft agar was measured as previously described (21). The number of transformed colonies was determined after 12 days. Transformation of primary rat embryo fibroblasts was carried out as described previously (46).

Growth factors were used at the following final concentrations: platelet-derived growth factor, 10 ng/ml; tetradecanoyl phorbol acetate, 200 nM; insulin, 1 μg/ml; transforming growth factor alpha, 20 ng/ml; isobutyl methyl xanthine, 100 μM; forskolin, 10 μM.

For transient transfection assays, $3 \times 10^5$ CV-1 cells were plated into 60-mm-diameter dishes. The next day, cells were refed with fresh medium. Four hours later, cells were transfected by calcium phosphate precipitation (66). At 48 h later, cells were harvested, extracts were prepared, and luciferase activity was measured as described previously (37). BALB/c-3T3 cells were plated at a density of $10^4$/60-mm dish and grown for 2 days before transfection. All transfections contained equal effector plasmid amounts. For each point, three independent transfections were performed.

**Northern (RNA) blotting, primer extension analysis, and runoff transcription assays.** Total RNA was prepared by the guanidium thiocyanate method (15). Samples (10 μg) of total RNA were separated by formaldehyde gel electrophoresis and transferred to Hybond $N^+$ membranes (Amersham) by capillary transfer (66). Expression of cyclin D1 was measured with a mouse cyl1 probe (48), and that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured with a rat cDNA probe (27). Primer extensions were carried out with 10 μg of total RNA from MS107 human diploid fibroblasts (54). The primer has the sequence GGCTCCAGGACTTTGCAACTTCAACAAAAC and hybridizes to nucleotides +90 to +61 in accordance with the numbering in Fig. 5. Runoff transcription assays were done as previously described (22); filters were analyzed with a phosphoimager, and transcription rates were calculated as counts per minute hybridizing specifically to the cyclin D1 probe relative to hybridizing to a GAPDH probe.

**Western blot (immunoblot) analysis and immunoprecipitations.** Immunoprecipitations were carried out with either preimmune antiserum or polyclonal anti-Max or anti-Myc serum (kind gift of G. Evan) essentially as described by Blackwood et al. (12). Immunoprecipitates or whole-cell extracts were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis, and the proteins were electrophoretically blotted onto polyvinylidene difluoride membranes. The membranes were then incubated with 5% skim milk–1% bovine serum albumin–0.01% gelatin–0.02% Tween 20 in Tris-buffered saline (pH 8) for 2 h and then with anti-human Myc monoclonal antibody 9E10 or 3C7 (23) in this buffer overnight at 4°C. The membranes were washed three times with Tris-buffered saline containing 0.01% Tween 20 and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (Dianova) for 3 h at room temperature. After the membranes were washed with Tris-buffered saline–0.01% Tween 20, immunolabelled bands were detected with the ECL Western blotting detection system (Amersham).

**Gel mobility shift analysis.** USF (partially purified from HeLa cells) was a kind gift of Vincent Montcollin (Institut National de la Santé et de la Recherche Médicale, Strasbourg, France); antibodies raised against recombinant USF were a kind gift of Michele Sawadogo. The cyclin D1 initiator sequence used was 5′-CCTCCCGCTCCCATTCTCTGCCGGG-3′, the adenovirus major late promoter Inr sequence used was 5′-GGCGCGTTCGTCCTCACTCTCTTCCGCATCGCTGTCTG-3′, the E-box sequence was 5′-ACCCGACCACGT

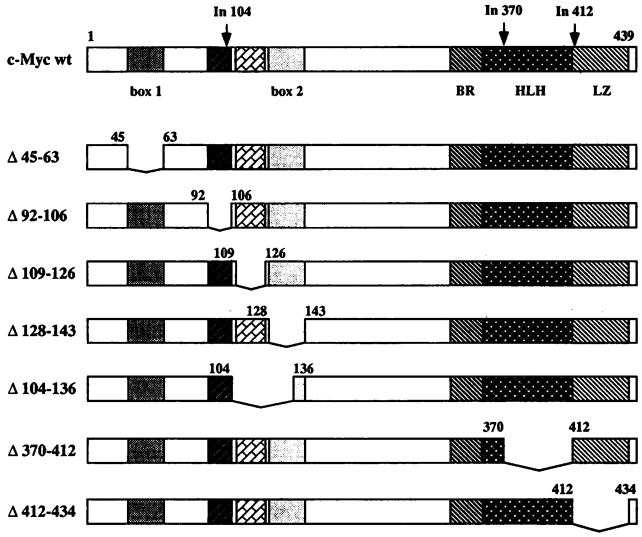


FIG. 1. Mutant proteins used in this study. The diagrams depict the wt human Myc protein and the mutant proteins that were expressed in BALB/c-3T3 cells. Boxes 1 and 2 represent regions of homology among all Myc proteins, BR is the basic region, HLH is the helix-loop-helix motif, and LZ is the leucine zipper. The numbers are those of the deleted amino acids or the positions at which insertions occurred.

GGTCTGAG-3′, and the mutated sequence E-box mut was 5′-ACCCGACTGAGTGGTCTGAG-3′. Gel shifts were carried out essentially as previously described (64).

## RESULTS

The functional domains of Myc involved in transformation have been well characterized (67, 75). To determine whether repression of cyclin D1 by Myc is an indirect consequence of cell transformation by Myc, we compared the functional domains involved in cyclin D1 repression to those involved in cell transformation. PCR was used to delete small, evolutionarily conserved domains in the amino terminus of Myc (Fig. 1). In addition, we included several mutants originally described by Stone et al. (75) in our study. In each case, the presence of the mutations was verified by sequencing and in vitro translation was used to demonstrate that proteins of the expected sizes were synthesized (data not shown and Fig. 2B and 3B).

Recombinant retroviruses carrying the mutant *MYC* genes were generated, and BALB/c-3T3 fibroblasts were infected, selected for G418-resistant colonies, and pooled. Several hundred colonies were observed after each infection. Expression of the exogenous *MYC* gene was verified both by Northern blotting (data not shown) and by Western blotting with either monoclonal antibody 9E10 or 3C7 (23) (Fig. 2B and 3B). The Northern blotting results suggest that the levels of exogenous *MYC* mRNA are between 5- and 10-fold higher than those of the endogenous gene (data not shown). Western blots (Fig. 2B and 3B) demonstrated that with the exception of mutant Δ45-63 (which appears to be unstable in vivo), all proteins were expressed at equal levels.

To determine levels of cyclin D1 mRNA expression in these cells, they were plated in 10% newborn calf serum at low density and allowed to grow for 48 h. At this time, RNA was harvested and the amounts of cyclin D1 and, as a control, GAPDH mRNAs were determined by Northern blotting. The results of this experiment are shown in Fig. 2A.

The results confirmed that BALB/c-3T3 cells that express

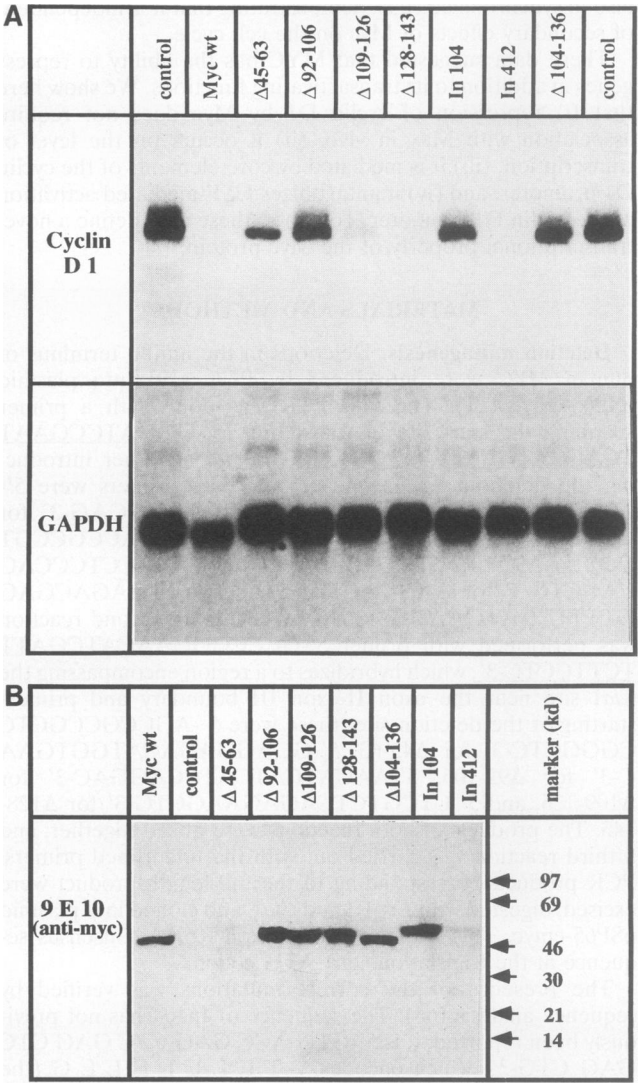


FIG. 2. Cyclin D1 expression in BALB/c-3T3 cells expressing amino-terminally mutated *MYC*. (A) Northern blot of cyclin D1 mRNA and GAPDH mRNA in exponentially growing BALB/c-3T3 cells carrying different alleles of *MYC*. (B) Western blot documenting expression of the human MYC protein with monoclonal antibody 9E10, which specifically recognizes the human protein. kd, kilodaltons.

human *MYC* contain significantly lower levels of cyclin D1 mRNA than those that do not (compare lanes 1 and 2). Of the mutant alleles, three were unable to repress cyclin D1: Δ92-106, In104, and Δ104-136. All of these have deletions or disruptions of the integrity of a small region centered around amino acid 104. One immediately adjacent deletion, Δ109-126, had levels of cyclin D1 mRNA almost equal to those found in cells expressing the wt *MYC* gene, thus defining a carboxy-terminal border for the domain involved in cyclin D1 repression. We conclude from these data that a small region centered around amino acid 104 is critically important for the ability of Myc to repress cyclin D1. The results also demonstrate that Myc homology box II (Δ128-143) is not involved in repression of cyclin D1. Also, an insertion mutant protein with a disruption in the spacing between the helix-loop-helix region and the leucine zipper (In412) was still able to repress cyclin D1. Western blotting with an affinity-purified anti-cyclin D1 poly-

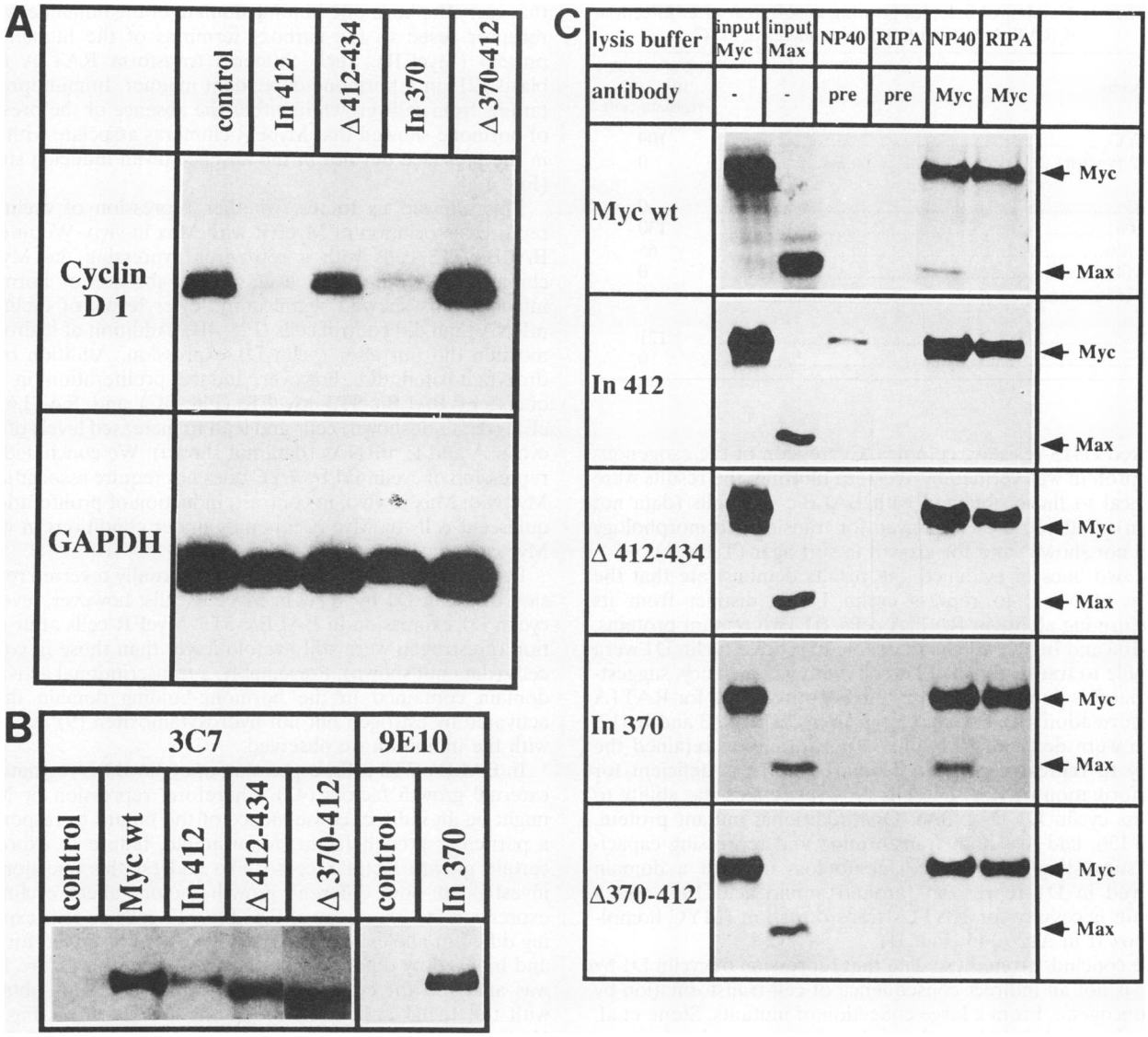


FIG. 3. Cyclin D1 expression in BALB/c-3T3 cells expressing carboxy-terminal mutant forms of *MYC*. (A) Northern blot of cyclin D1 mRNA and GAPDH mRNA in exponentially growing BALB/c-3T3 cells carrying the indicated alleles of *MYC*. (B) Western blot analysis documenting expression of the mutant alleles. Cellular lysates were immunoprecipitated with polyclonal anti-Myc serum; immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted with monoclonal antibody 3C7. (C) In vitro association between mutant alleles of Myc and Max. The indicated alleles of Myc were synthesized in a reticulocyte lysate in the presence of [$^{35}$S]methionine, mixed with in vitro-translated Max, and immunoprecipitated either in the presence (RIPA) or in the absence (NP40) of an ionic detergent with a polyclonal anti-Myc antibody. Control immunoprecipitations contained preimmune serum (pre). NP40, Nonidet P-40.

clonal antibody (kind gift of Giulio Draetta) demonstrated that protein levels parallel mRNA levels in these cells (data not shown).

To determine more precisely if domains of the carboxy terminus of Myc are required for repression of cyclin D1, three additional mutant proteins originally described by Stone et al. (75) were analyzed as described above: In370, with an insertion of four amino acids at the start of helix 1 of the helix-loop-helix motif; Δ370-412, with a deletion of the helix-loop-helix domain; and Δ412-434, with a deletion of the leucine zipper (Fig. 1). Expression of the genes for these mutant proteins (except In370) after infection of BALB/c-3T3 cells was verified by immunoprecipitation with a polyclonal anti-Myc antibody followed by Western blotting with monoclonal antibody 3C7 (23) (Fig. 3B). Cyclin D1 levels in the infected cells are shown in Fig. 3A. Of these mutant proteins, Δ370-412 completely lost the ability to repress cyclin D1, showing that the helix-loop-helix domain of Myc is required for repression. In370 was, however, still fully competent for repression, suggesting that precise alignment of basic regions in a heterodimer may not be required for repression of cyclin D1 by MYC. Deletion of the entire leucine zipper led to partial loss of repression: this mutant yielded variable results in several experiments (Fig. 3A and data not shown), demonstrating that the requirement for the integrity of the leucine zipper is not absolute.

To compare these results to the transforming properties of the different alleles of *MYC*, we infected RAT1A fibroblasts with retroviruses carrying the mutated alleles of *MYC* and

TABLE 1. Number of colonies growing in soft agar after infection of RAT1A cells with different alleles of *MYC*

| Virus | No. of colonies/ $10^3$ G418-resistant cells |
|---|---|
| wt MYC | 109 |
| pMV7 (vector) | 0 |
| Δ45-63 | 0 |
| Δ92-106 | 130 |
| Δ109-126 | 65 |
| Δ128-143 | 0 |
| Δ104-136 | 0 |
| In104 | 121 |
| In412 | 0 |

selected G418-resistant colonies. Expression of the exogenous Myc protein was verified by Western blotting: the results were identical to those obtained with BALB/c-3T3 cells (data not shown). Cells were then assayed for transformed morphology (data not shown) and for growth in soft agar (Table 1).

By two lines of evidence, the results demonstrate that the ability of MYC to repress cyclin D1 is distinct from its transforming ability in RAT1A cells. (i) Two mutant proteins, Δ92-106 and In104, which are unable to repress cyclin D1 were still able to transform RAT1A cells with wt efficiency, suggesting that this domain of the protein is not required for RAT1A transformation. (ii) Two mutant proteins, Δ128-143 and In412, which were deficient in RAT1A transformation retained the ability to repress cyclin D1. Similarly, In370 is deficient for transformation of RAT1A cells (75) yet retains the ability to repress cyclin D1 (Fig. 3A). One additional mutant protein, Δ104-136, had lost both transforming and repressing capacities, suggesting that this is due to loss of both a domain involved in D1 repression (around amino acid 104) and a domain necessary for RAT1A transformation (MYC homology box II in Δ128-143 [Fig. 1]).

We conclude from these data that repression of cyclin D1 by *MYC* is not an indirect consequence of cell transformation by this oncogene. From a large collection of mutants, Stone et al. (75) identified In104 as the only mutant protein that was specifically unable to transform primary rat embryo fibroblasts in cooperation with EJ-*ras* but retained the ability to transform established RAT1A cells. Cotransfection of mutant alleles of *MYC* together with EJ-*ras* confirmed this observation for In104 and showed that a second mutation in the same region Δ92-106) causes a similar phenotype (data not shown), suggesting that the domain involved in repression of cyclin D1 is specifically required for transformation of primary cells.

Transformation of RAT1A cells by Myc critically depends on the ability of the protein to heterodimerize with Max (2, 4). Therefore, we wondered whether association of Max with Myc is also required for repression of cyclin D1 by Myc. Alleles of Myc that carried mutations in the carboxy terminus were synthesized in the reticulocyte lysate, mixed with the in vitro-translated Max protein, and immunoprecipitated with a polyclonal anti-Myc antibody in the presence of either nonionic or ionic detergent. The results (Fig. 3C) showed that with the exception of In370, none of the C-terminal mutant proteins was capable of interacting with Max in vitro. As In412 is fully capable of repressing cyclin D1 in vivo, this observation suggested that repression of cyclin D1 is not mediated by the Myc-Max complex.

To test this hypothesis in vivo, we used chimeric proteins that carry the hormone-binding domain of the human estrogen receptor fused to the carboxy terminus of the human Myc protein (MycER). Such chimeras transform RAT1A fibroblasts (21) in a hormone-dependent manner. Immunoprecipitations from cells grown in either the absence or the presence of hormone showed that MycER chimeras associate with Max in the presence but not in the absence of an inducing steroid (Fig. 4A).

This allowed us to test whether repression of cyclin D1 requires association of MycER with Max in vivo. We infected BALB/c-3T3 cells with a retrovirus expressing the MycER chimera and found that even in the absence of hormone, infected cells showed significantly lower levels of cyclin D1 mRNA than did control cells (Fig. 4B). Addition of hydroxytamoxifen did not alter cyclin D1 expression. Addition of hydroxytamoxifen did, however, induce proliferation in both quiescent BALB/c-3T3–MycER (Fig. 4C) and RAT1A-MycER (data not shown) cells and lead to increased levels of both cyclin A and E mRNAs (data not shown). We concluded that repression of cyclin D1 by *MYC* does not require association of Myc with Max in vivo; in contrast, induction of proliferation in quiescent cells by Myc occurs only under conditions in which Myc can associate with Max.

Paradoxically, addition of estrogen partially reverted repression of cyclin D1 by *MYC* in MycER cells: however, levels of cyclin D1 expression in BALB/c-3T3–MycER cells after addition of estrogen were still fivefold lower than those in control cells (data not shown). Presumably, a transcriptional activation domain contained in the hormone-binding domain that is activated by estrogen but not hydroxytamoxifen (9) interferes with the inhibition we observed.

In BALB/c-3T3 cells, expression of cyclin D1 is regulated by external growth factors (43). Therefore, repression by MYC might be an indirect consequence of the failure to respond to a particular growth factor, for example, failure to express a certain growth factor receptor. To address this question, we investigated how different growth factors affect cyclin D1 expression in either control BALB/c-3T3 cells or cells expressing different alleles of *MYC*. Cells were serum starved for 48 h and induced by different growth factors for 10 h before RNA was analyzed for cyclin D1 expression. The results obtained with the In104 and In412 mutations are shown in Fig. 5A. Cyclin D1 was induced by serum, tetradecanoyl phorbol acetate, transforming growth factor alpha, platelet-derived growth factor, and insulin in both vector-infected control (data not shown) and In104 (Fig. 5) cells, confirming results obtained with human fibroblasts (82). None of the growth factors able to induce cyclin D1 in cells carrying the In104 allele of *MYC* was able to do so in In412-carrying cells (Fig. 5A). Similar results were obtained with cells expressing wt *MYC* (data not shown). Control experiments showed that neither allele interfered with the ability of serum to induce proliferation and expression of the endogenous c-*fos* gene (data not shown). Repression of cyclin D1 is therefore not an indirect consequence of the failure to respond to a particular growth factor critical for induction of cyclin D1.

Runoff experiments have shown that induction of cyclin D1 mRNA by serum growth factors occurs at the level of transcription initiation (43). We wondered whether expression of *MYC* blocked this increase and performed runoff experiments with control and In412-carrying cells before and 2 h after addition of serum to growth factor-deprived cells. The results (Fig. 5B) demonstrated that expression of *MYC* inhibits growth factor-induced transcription of the cyclin D1 gene. We conclude that *MYC* represses cyclin D1 at the transcriptional level.

To analyze the mechanism of repression by MYC, we

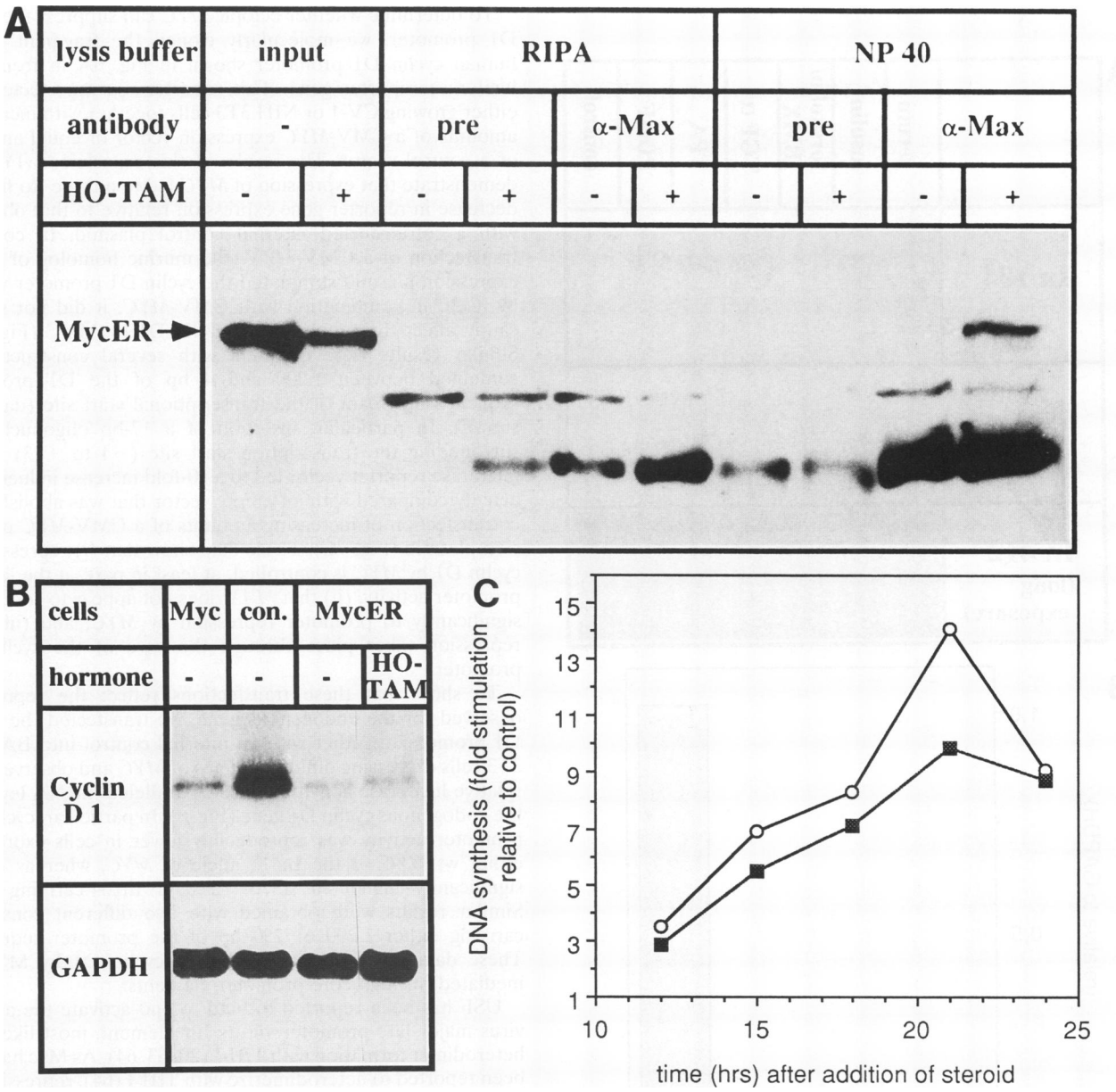


FIG. 4. Repression of cyclin D1 by Myc does not require association with Max in vivo. (A) Max and MycER associate in a hormone-dependent manner. Shown is a Western blot of MycER proteins present in whole-cell extracts (input) or in immunoprecipitates from RAT1A-MycER cells grown either in hormone-free medium or in the presence of 250 nM hydroxytamoxifen (HO-TAM), as indicated. The lysis buffer and antiserum used are indicated above the lanes. The band corresponding to MycER protein is indicated; the two lower bands correspond to the heavy and light chains of the antibody used for immunoprecipitation. The MycER protein was specifically detected in anti-Max immunoprecipitates only in the presence of hydroxytamoxifen. NP40, Nonidet P-40. (B) Northern blot of cyclin D1 and GAPDH mRNAs in exponentially growing BALB/c-3T3 cells. Where indicated, cells were treated with 250 nM hydroxytamoxifen for several days before the experiment. con., control. (C) [$^3$H]thymidine incorporation of BALB/c-3T3–MycER cells treated with 200 nM estrogen (■) or 250 nM hydroxytamoxifen (○) relative to that of untreated control cells. Cells were serum starved for 48 h before the experiment.

isolated the promoter of the human cyclin D1 gene from a genomic library. Figure 6A shows the sequence of a promoter fragment extending 360 bp 5′ of the end of the human cDNA clone. This fragment showed promoter activity in transient transfection assays with CV-1 cells (data not shown). The promoter lacks consensus TATA and CCAAT elements and contains binding sites for (among other potential sites) Egr (68a) and E2F (31a). To determine the transcription start site, primer extension analysis was carried out with RNA isolated from MS107 diploid human fibroblasts (54) (Fig. 6B). Two major start sites were found at immediately adjacent C residues; these are located 40 bp upstream of the 5′ end of the mouse cDNA clone. In addition, two very minor start sites were detected at positions −3 and +6 relative to the major start sites. The sequence surrounding the major start sites shows strong sequence homology to the Inr elements of the adenovirus major late, the terminal deoxynucleotide transferase, the N-CAM, and the CD44 promoters (Fig. 6C).

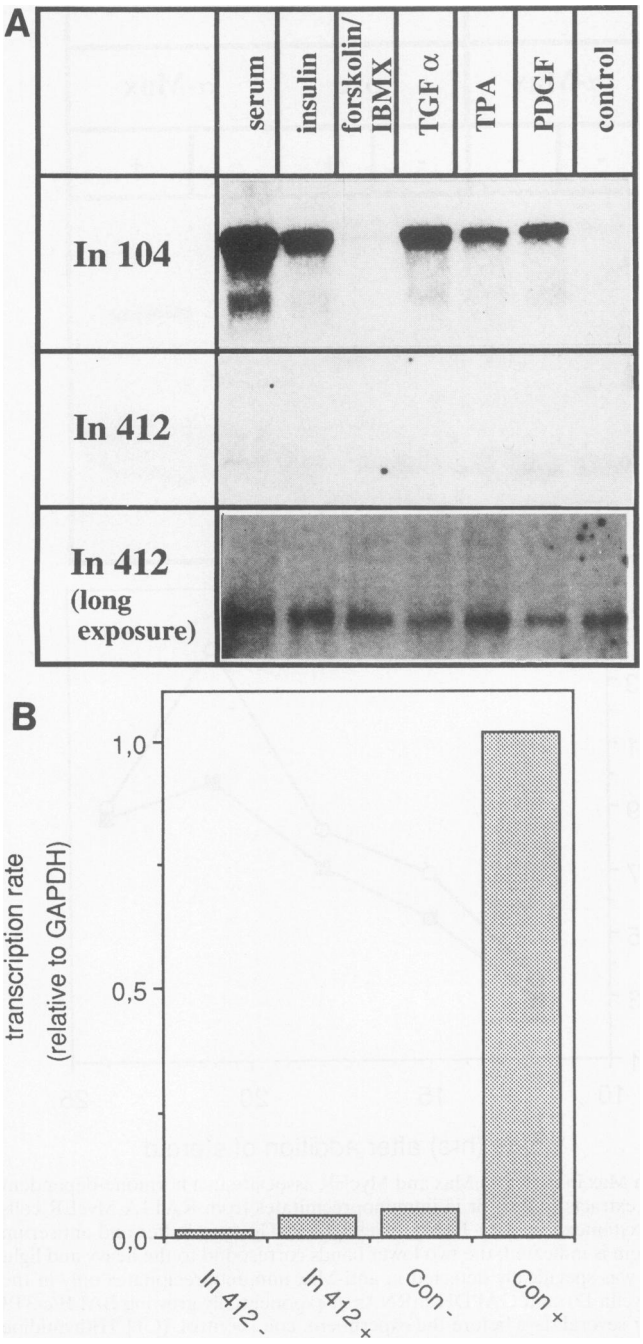


FIG. 5. Regulation of cyclin D1 expression by external growth factors in BALB/c-3T3 cells expressing the In104 and In412 alleles of *MYC*. (A) BALB/c-3T3 cells carrying either the In412 or the In104 allele of *MYC* were serum starved for 48 h in medium containing 0.2% newborn calf serum and then stimulated with the indicated growth factors for 10 h before RNA was harvested and analyzed for cyclin D1 expression. TPA, tetradecanoyl phorbol acetate; PDGF, platelet-derived growth factor; TGFα, transforming growth factor alpha; IBMX, isobutyl methyl xanthine. (B) Runoff transcription assays from vector control (pMV7) and In412 cells; shown are transcription rates before (−) and 2 h after (+) serum stimulation of cyclin D1 relative to GAPDH. Con, control.

To determine whether ectopic *MYC* can suppress the cyclin D1 promoter, we molecularly cloned the fragment of the human cyclin D1 promoter shown in Fig. 6A in front of a luciferase reporter gene. This reporter was transfected into either growing CV-1 or NIH 3T3 cells together with increasing amounts of a CMV-*MYC* expression vector or equal amounts of a control vector. The results of this experiment (Fig. 7A) demonstrate that expression of *MYC* led to a three- to fivefold decrease in reporter gene expression relative to that obtained with a cotransfected internal control plasmid. In contrast, transfection of a CMV-*MYN* (the murine homolog of *MAX*) expression plasmid stimulated the cyclin D1 promoter weakly by itself; in combination with CMV-*MYC*, it did not significantly affect the repression of cyclin D1 by MYC (Fig. 7B). Similar results were obtained with several constructs that contained between 1,200 and 4 bp of the D1 promoter sequence upstream of the transcriptional start site (data not shown). In particular, insertion of a 17-bp oligonucleotide surrounding the transcription start site (−4 to +13) into a luciferase reporter vector led to a 10-fold increase in luciferase activity compared with an empty vector that was abolished by cotransfection of increasing amounts of a CMV-*MYC* expression plasmid (Fig. 7C). These data show that (i) repression of cyclin D1 by *MYC* is controlled, at least in part, at the level of promoter activity, (ii) that *MAX* does not appear to contribute significantly to promoter repression by *MYC*, and (iii) that repression takes place through the core of the cyclin D1 promoter.

To show that these transfections reflect the repression observed for the endogenous gene, we transfected the cyclin D1 promoter together with an internal control into BALB/c-3T3 cells expressing different alleles of *MYC* and observed that relative luciferase activities closely paralleled mRNA levels of the endogenous cyclin D1 gene (Fig. 8). In particular, cyclin D1 promoter activity was reproducibly lower in cells expressing either wt *MYC* or the In412 allele of *MYC*, whereas it was significantly higher in Δ370-412- and In104-carrying cells. Similar results were obtained with two different constructs carrying either 1,200 or 250 bp of the promoter sequence. These data show that repression of cyclin D1 by MYC is mediated through core promoter elements.

USF has been reported to bind to and activate the adenovirus major late promoter via its Inr element, most likely via heterodimer formation with TfII-I (20, 63, 64). As Myc has also been reported to heterodimerize with TfII-I (64), repression by MYC might result from inhibition of USF-mediated transactivation. Cotransfection of expression plasmids for USF and cyclin D1 reporter plasmids into HeLa cells resulted in 20- to 40-fold activation of the promoter of the cyclin D1 gene relative to a cotransfected Rous sarcoma virus β-galactosidase expression plasmid (Fig. 9A). Activation by USF is mediated through the first 250 bp of the promoter, which do not contain an E-box element (Fig. 6A). Coexpression of *MYC* inhibited activation by USF in a dose-dependent manner (Fig. 9A).

To test whether USF binds directly to the Inr element of the cyclin D1 promoter, gel shift experiments with partially purified USF (from HeLa cells; kind gift of Vincent Moncollin) were performed with a labelled oligonucleotide corresponding to the transcription start site. The results (Fig. 9B) showed that USF binds to the Inr element; competition within the unlabelled D1 Inr, the Inr element of the adenovirus major late promoter, and a consensus E-box element demonstrated that USF binds in a sequence-specific manner. Specific antibodies raised against USF (kind gift of Michele Sawadogo) supershifted the indicated band, confirming that the band indeed

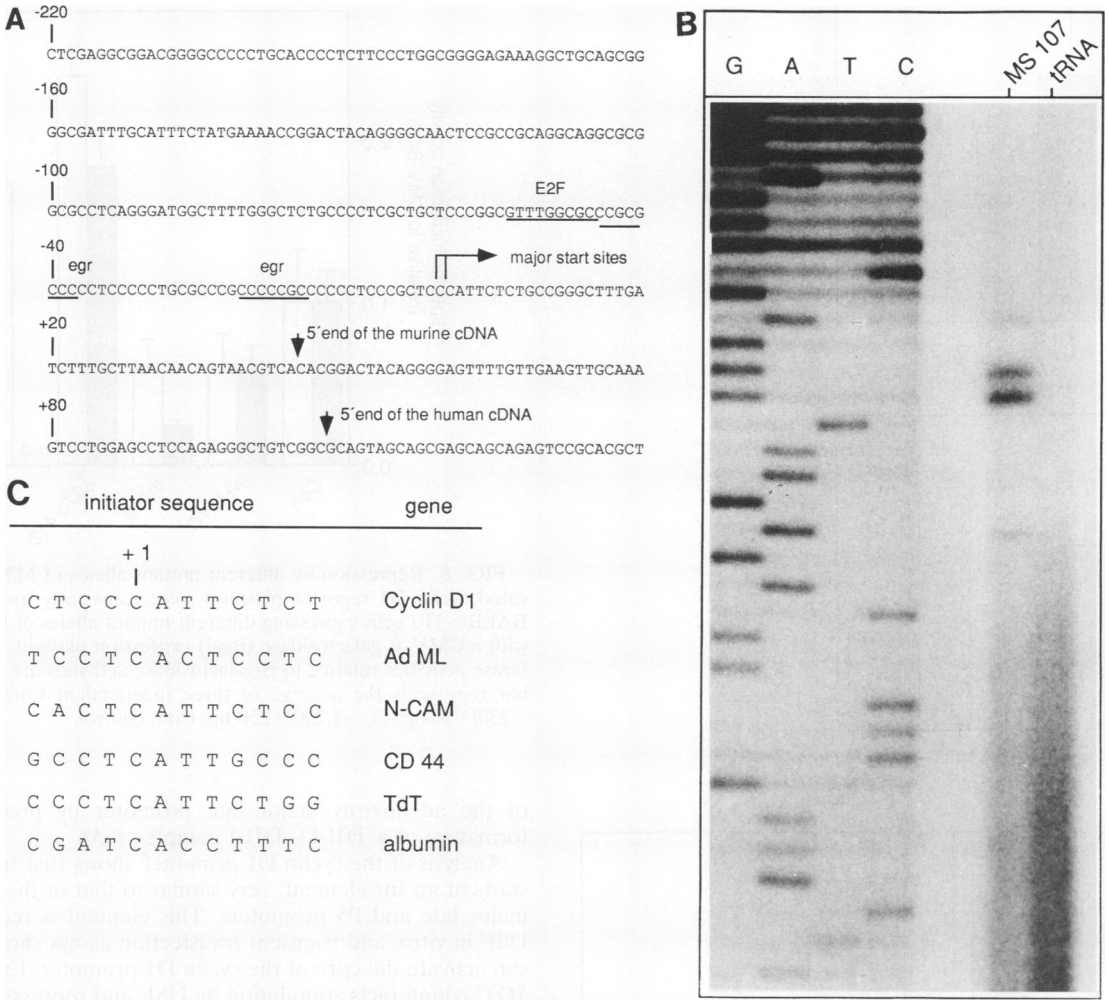


FIG. 6. Structure of the promoter of the human cyclin D1 gene. (A) Sequence of a 360-bp fragment upstream of the 5′ ends of the human and mouse cDNA molecules. The positions of the major transcription start in MS107 fibroblasts and of the Egr and E2F sites are indicated. (B) Primer extension analysis documenting the two major and two minor transcription start sites. (C) Alignment of Inr sequences from several genes. The Inr elements of the adenovirus major late promoter (Ad ML) have been demonstrated to interact with USF, TfII-I, and Myc. TdT, deoxynucleotide transferase.

contained USF (Fig. 9C). These results showed that USF can bind to and activate the core promoter of the cyclin D1 gene; they also demonstrated that MYC counteracts stimulation by USF.

## DISCUSSION

In this report, we document a novel property of the *MYC* oncogene: we show that the protein it encodes, Myc, can repress transcription of the cyclin D1 gene via core elements of the cyclin D1 promoter. Previously, Myc has been demonstrated to activate transcription from E-box sequences in conjunction with a partner protein, Max (3, 5, 11, 42). Four lines of evidence show that repression of cyclin D1 by Myc is not mediated by the Myc-Max complex. (i) The analysis of mutant forms of Myc shows that the ability of MYC to repress cyclin D1 is distinct from its ability to transform established RAT1A cells, which critically depends on heterodimerization with Max (2, 4). (ii) Expression of an insertion mutant (In412) form of Myc that fails to associate with Max in vitro still represses cyclin D1. (iii) In MycER chimeras, repression of cyclin D1 is a constitutive property although association with Max is hormone dependent. (iv) Ectopic Max has no effect on the cyclin D1 promoter and does not affect repression of this promoter by Myc.

The idea that Myc forms complexes with proteins other than Max is supported by two lines of evidence. (i) Activation by *MYC* of the two E-box sequences localized in the first intron of the ornithine decarboxylase gene is not affected by deletion of the leucine zipper. As this deletion abolishes association of Myc with Max, activation of ornithine decarboxylase appears to be mediated by a Myc complex other than the Myc-Max complex (7). (ii) Myc has been shown to heterodimerize with two proteins, TfII-I (63) and YY-1 (71), that are capable of binding to and activating transcription from Inr sequences of the adenovirus major late promoter (64) and the adeno-associated virus type 2 P5 promoter (69), respectively. At the adenovirus major late promoter Inr element, Myc appears to counteract the effect of USF, which has been shown to bind to and activate the adenovirus major late promoter via this element (20), most likely also as a heterodimer with TfII-I (64). In vitro, Myc mediates repression from the Inr sequence

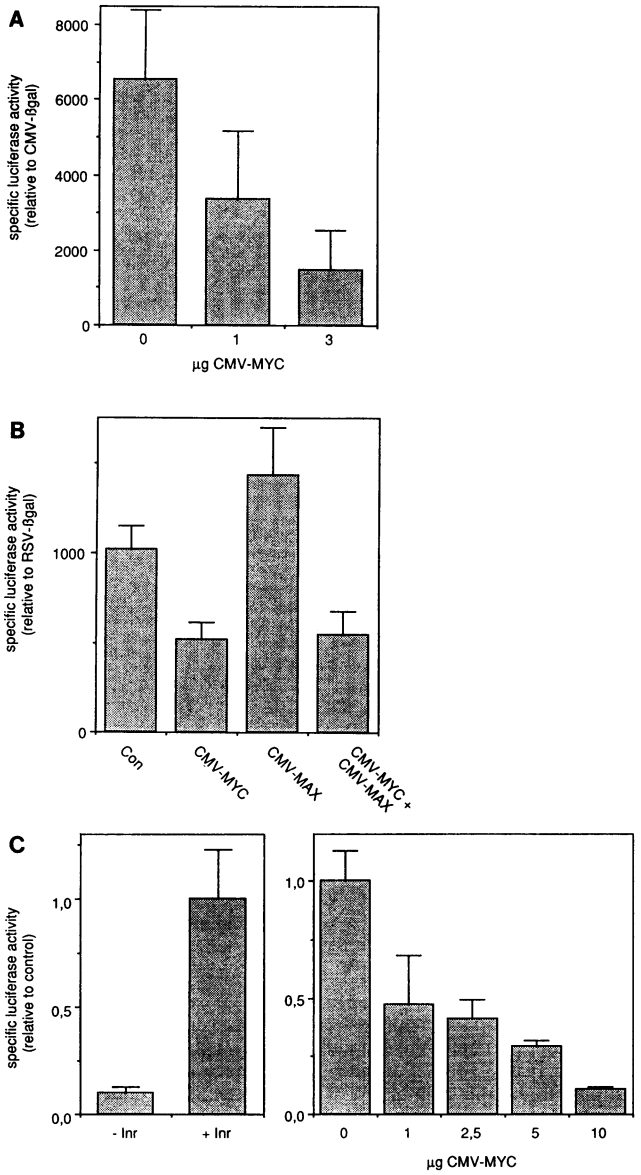


FIG. 7. Repression of cyclin D1 promoter in transient transfection assays. (A) Dose-dependent repression of the cyclin D1 promoter in NIH 3T3 cells. Cells were transfected with a reporter construct containing 250 bp of the human cyclin D1 promoter and either a CMV-*MYC* expression vector or equimolar amounts of a CMV-Myn or control CMV expression vector by a standard calcium phosphate technique. Shown is the specific luciferase activity (light units per microgram of protein) relative to β-galactosidase activities. Each bar represents the average of three independent transfections. Absolute light units were $5 \times 10^5$ per $5 \times 10^5$ cells in the absence of CMV-*MYC* and $5 \times 10^3$ per $5 \times 10^5$ cells in the presence of 3 μg of CMV-*MYC*. RSV, Rous sarcoma virus. (B) Expression of Max does not affect expression of the cyclin D1 promoter. CV-1 cells were transfected with CMV-*MAX* or CMV-*MYC* and analyzed as described above. Con, control. (C) The Inr element of the cyclin D1 promoter is sufficient to mediate repression by *MYC*. The left panel shows the basal activity of the Inr element. Shown is the specific luciferase activity after transfection of either an empty vector (−Inr) or a reporter vector containing the Inr element (+Inr) inserted upstream of the luciferase gene. CV-1 cells were transfected with increasing amounts of a CMV-*MYC* expression vector and a reporter containing only the Inr element. Shown is the specific luciferase activity (light units per microgram of protein) relative to that of a control transfection. Each bar represents the average of three independent transfections.

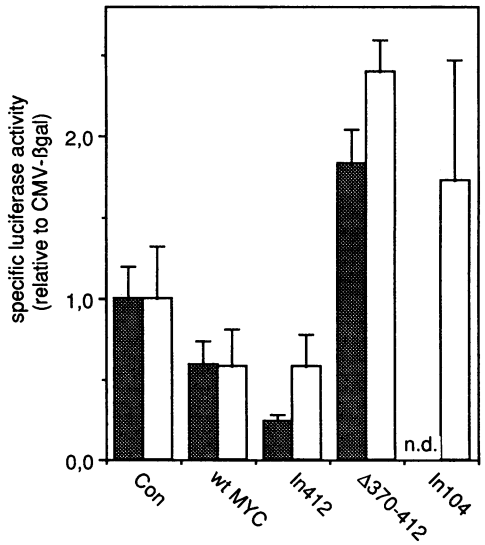


FIG. 8. Repression by different mutant alleles of MYC. The indicated cyclin D1 reporter plasmids were transiently transfected into BALB/c-3T3 cells expressing different mutant alleles of Myc together with a CMV–β-galactosidase (βgal) expression plasmid. Specific luciferase activities relative to β-galactosidase activities are shown. Each bar represents the average of three independent transfections. ▩, −250/+10 bp; □, −1,200/+250 bp. Con, control.

of the adenovirus major late promoter by precluding the formation of a TfII-D–TfII-I complex (63).

Analysis of the cyclin D1 promoter shows that transcription starts at an Inr element, very similar to that of the adenovirus major late and P5 promoters. This element is recognized by USF in vitro, and transient transfection assays show that USF can activate the core of the cyclin D1 promoter. Expression of *MYC* counteracts stimulation by USF and represses cyclin D1 through the core of the promoter; the Inr element is sufficient to mediate repression by *MYC*. Most likely, therefore, interactions at this element contribute to repression of cyclin D1 by *MYC* in vivo. Preliminary experiments show that TfII-I stimulates expression of the cyclin D1 promoter and that *MYC* abolishes this transactivation (68b). Our data do not, however, rule out the possibility that other protein-protein interactions contribute to repression by *MYC*: for example, YY-1 is the major Inr-binding protein in HeLa and BALB/c-3T3 extracts (68a). Further work is necessary to resolve this issue.

Several genes that are repressed by *MYC* have been described, including c-*myc* itself (55), the N-CAM-encoding gene (1), the *neu* proto-oncogene (76), collagen genes (83), and more recently, the c/EBP-α and albumin genes (43a). In addition, repression of CD44 in cells that express high levels of N-Myc or c-Myc has been reported (72). Of these, c/EBP-α, albumin, CD44, and N-CAM use Inr sequences similar to those described above. Repression of Inr elements by *MYC* may, therefore, contribute significantly to the changes in gene expression observed in *MYC*-transformed cells.

Critical for repression of cyclin D1 by *MYC* is a small domain in the amino terminus of the protein. Data base searches show that this domain has structural similarity to a domain (HOB2) necessary for the transactivating function of c/EBP, Fos, and Jun (77) and to the amino terminus of E1A (data not shown). Indeed, we have found that this region can functionally replace the HOB2 domain in chicken c/EBP-β (41a). Similar to Myc, the adenovirus E1A protein has been reported to repress

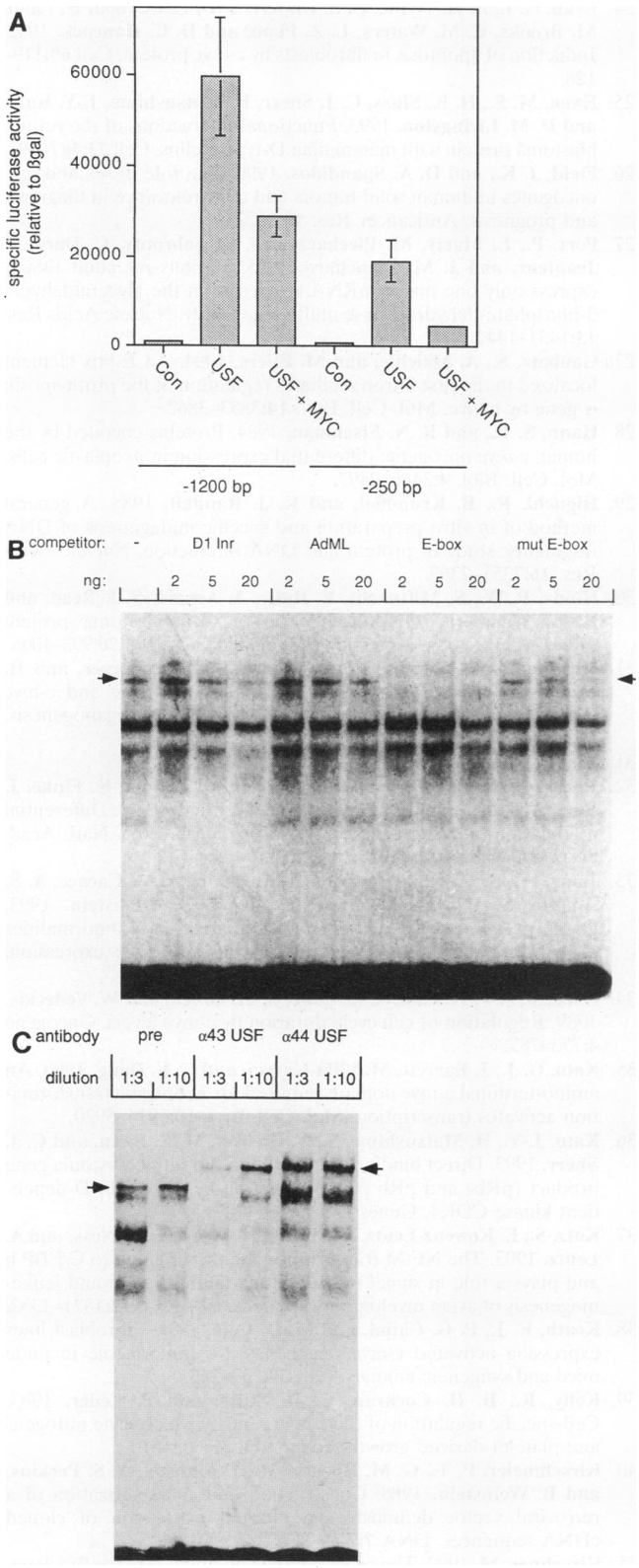


FIG. 9. USF binds to and transactivates the core of the cyclin D1 promoter. (A) Transient transfection assays into HeLa cells with the indicated cyclin D1 reporter plasmids and either a control expression plasmid (Con), 10 μg of CMV-USF (USF), or 10 μg of CMV-MYC and 3 μg of CMV-MYC (MYC + USF). βgal, β-galactosidase. (B) Specific binding of USF to the cyclin D1 Inr element. Partially purified USF was incubated with a labelled oligonucleotide spanning the cyclin D1 Inr element either in the absence (−) or in the presence of the indicated competitor oligonucleotides. AdML, adenovirus major late promoter. (C) Anti-USF antibodies recognize a complex specifically binding to the cyclin D1 Inr element. A gel mobility shift experiment was performed as described before; after the binding reaction, the samples were incubated with the indicated dilutions of either preimmune serum (pre) or two different polyclonal sera raised against the 43- and 44-kDa forms of USF (68). The arrows indicate the complex containing USF.

expression of cyclin D1 (14, 74); transient transfection assays showed that the amino terminus of E1A is involved in cyclin D1 repression (74a). It is conceivable, therefore, that this motif mediates a protein-protein contact essential for repression of cyclin D1 that is common to both Myc and E1A.

Expression of cyclin D1 can be induced by growth factors in quiescent cells (43, 48, 82). However, its expression often does not correlate with the proliferative status of the cell. For example, expression of cyclin D1 is induced in response to the retinoblastoma protein (30, 52) and is also induced during senescence of human fibroblasts (45). In particular, it is striking that cyclin D1 is downregulated in response to at least three nuclear oncogenes, those that encode adenovirus E1A (14, 74), simian virus 40 large T (45a, 52), and MYC (32). Also, Myc may affect translation of cyclin D1 (62). In the normal cell cycle, the cyclin D1 protein is present during the $G_1$ phase and disappears as cells start to replicate their DNA (6). Injection of the recombinant cyclin D1 protein leads to an arrest at the $G_1$-S boundary, suggesting that destruction of cyclin D1 is essential for cell cycle progression (53a). Also, a high level of cyclin D1 expression inhibits cell proliferation (61). It is tempting to speculate, therefore, that the decrease in cyclin D1 levels caused by nuclear oncogenes removes a rate-limiting step at the $G_1$-S boundary. Support for this idea comes from the observation that BALB/c-3T3 cells that carry alleles of *MYC* capable of repressing cyclin D1 have a higher percentage of cells in the S phase than those that do not, whereas cells that carry alleles of Myc unable to repress cyclin D1 show a delayed progression from mitosis to DNA replication compared with control cells (56a).

However, we do not know how many and which other genes are regulated via this pathway, and as a consequence, our studies do not directly address the role of cyclin D1 in transformation and immortalization by *MYC*. The alterations in transformation and proliferation that we observed probably reflect a number of genetic changes that are induced by *MYC*. Nevertheless, our data define a new pathway of gene regulation by MYC.

## ACKNOWLEDGMENTS

This work was supported by the Bundesministerium für Forschung und Technologie (grant BCT0381-5) and the HFSPO. A.P. acknowledges receipt of a fellowship from BASF Aktiengesellschaft. A.S. is supported by a fellowship from the Boehringer Ingelheim Foundation.

We thank Oliver Aalami for help with the gel mobility shift experiments.

## REFERENCES


1. **Akeson, R., and R. Bernards.** 1990. N-*myc* down regulates neural cell adhesion molecule expression in rat neuroblastoma. Mol. Cell. Biol. **10**:2012–2016.
2. **Amati, B., M. W. Brooks, N. Levy, T. D. Littlewood, G. I. Evan, and H. Land.** 1993. Oncogenic activity of the c-Myc protein requires dimerization with Max. Cell **72**:233–245.
3. **Amati, B., S. Dalton, M. W. Brooks, T. D. Littlewood, G. I. Evan, and H. Land.** 1992. Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max. Nature (London) **359**:423–426.
4. **Amati, B., T. D. Littlewood, G. I. Evan, and H. Land.** 1993. The c-Myc protein induces cell cycle progression and apoptosis through dimerization with Max. EMBO J. **13**:5083–5087.
5. **Amin, C., A. J. Wagner, and N. Hay.** 1993. Sequence-specific transcriptional activation by Myc and repression by Max. Mol. Cell. Biol. **13**:383–390.
6. **Baldin, V., J. Lukas, M. J. Marcote, M. Pagano, and G. Draetta.** 1993. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev. **7**:812–821.
7. **Bello-Fernandez, C., G. Packham, and J. L. Cleveland.** 1993. The ornithine decarboxylase gene is a transcriptional target of c-MYC. Proc. Natl. Acad. Sci. USA **90**:7804–7808.
8. **Benvenisty, N., A. Leder, A. Kuo, and P. Leder.** 1992. An embryonically expressed gene is a target for c-Myc regulation via the c-Myc-binding sequence. Genes Dev. **6**:2513–2524.
9. **Berry, M., D. Metzger, and P. Chambon.** 1990. Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxy-tamoxifen. EMBO J. **9**:2811–2818.
10. **Blackwell, T. K., L. Kretzner, E. M. Blackwood, R. N. Eisenman, and H. Weintraub.** 1990. Sequence-specific DNA binding by the c-myc protein. Science **250**:1149–1151.
11. **Blackwood, E. M., and R. N. Eisenman.** 1991. Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with myc. Science **251**:1211–1217.
12. **Blackwood, E. M., B. Lüscher, and R. N. Eisenman.** 1992. Myc and Max associate in vivo. Genes Dev. **6**:71–80.
13. **Brown, A. M. C., and M. R. D. Scott.** 1988. Retroviral vectors, p. 189–212. *In* D. Glover (ed.), DNA cloning, vol. III. IRL Press, Oxford.
14. **Buchou, T., O. Kranenburg, H. van Dam, D. Roelen, A. Zantema, F. L. Hall, and A. van der Eb.** 1993. Increased cyclin A and decreased cyclin D levels in adenovirus 5 E1A-transformed rodent cell lines. Oncogene **8**:1765–1773.
15. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
16. **Colby, W. W., E. Y. Chen, D. H. Smith, and A. D. Levinson.** 1983. Identification and nucleotide sequence of a human locus homologous to the v-*myc* oncogene of avian myelocytomatosis virus MC29. Nature (London) **301**:722–725.
17. **Deuschle, U., R. Pepperkok, F. B. Wang, T. J. Giordano, W. T. McAllister, W. Ansorge, and H. Bujard.** 1989. Regulated expression of foreign genes in mammalian cells under the control of coliphage T3 RNA polymerase and lac repressor. Proc. Natl. Acad. Sci. USA **86**:5400–5404.
18. **Dowdy, S. F., P. W. Hinds, K. Louie, S. I. Reed, A. Arnold, and R. A. Weinberg.** 1993. Physical interaction of the retinoblastoma protein with human D cyclins. Cell **73**:499–511.
19. **Downs, K. M., G. R. Martin, and J. M. Bishop.** 1989. Contrasting patterns of c-myc and N-myc expression during gastrulation of the mouse embryo. Genes Dev. **3**:860–869.
20. **Du, H., A. L. Roy, and R. G. Roeder.** 1993. Human transcription factor USF stimulates transcription through the initiator elements of the HIV-1 and the Ad-ML promoters. EMBO J. **12**:501–511.
21. **Eilers, M., D. Picard, K. Yamamoto, and J. M. Bishop.** 1989. Chimaeras between the MYC oncoprotein and steroid receptors cause hormone-dependent transformation of cells. Nature (London) **340**:66–68.
22. **Eilers, M., S. Schirm, and J. M. Bishop.** 1991. The MYC protein activates transcription of the alpha-prothymosin gene. EMBO J. **10**:133–141.
23. **Evan, G. I., G. K. Lewis, G. Ramsay, and J. M. Bishop.** 1985. Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. Mol. Cell. Biol. **5**:3610–3616.
24. **Evan, G. I., A. H. Wyllie, C. S. Gilbert, T. D. Littlewood, H. Land, M. Brooks, C. M. Waters, L. Z. Penn, and D. C. Hancock.** 1992. Induction of apoptosis in fibroblasts by c-myc protein. Cell **69**:119–128.
25. **Ewen, M. E., H. K. Sluss, C. J. Sherr, H. Matsushime, J.-Y. Kato, and D. M. Livingston.** 1993. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell **73**:487–497.
26. **Field, J. K., and D. A. Spandidos.** 1990. The role of *ras* and *myc* oncogenes in human solid tumors and their relevance in diagnosis and prognosis. Anticancer Res. **10**:1–22.
27. **Fort, P., L. Marty, M. Piechaczyk, S. El Sabrouty, C. Dani, P. Jeanteur, and J. M. Blanchard.** 1985. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. Nucleic Acids Res. **13**:1431–1442.

27a. **Gaubatz, S., A. Meichle, and M. Eilers.** 1994. An E-box element localized in the first intron mediates regulation of the prothymosin α gene by c-*myc*. Mol. Cell. Biol. **14**:3853–3862.

28. **Hann, S. R., and R. N. Eisenman.** 1984. Proteins encoded by the human c-*myc* oncogene: differential expression in neoplastic cells. Mol. Cell. Biol. **4**:2486–2497.
29. **Higuchi, R., B. Krummel, and K. J. Randall.** 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interaction. Nucleic Acids Res. **16**:7351–7367.
30. **Hinds, P. W., S. Mittnacht, V. Dulic, A. Arnold, S. I. Reed, and R. A. Weinberg.** 1992. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell **70**:993–1006.
31. **Hirning, U., P. Schmid, W. A. Schulz, G. Rettenberger, and H. Hameister.** 1991. A comparative analysis of N-myc and c-myc expression and cellular proliferation in mouse organogenesis. Mech. Dev. **33**:119–125.

31a. **Jansen-Dürr, P.** Unpublished data.

32. **Jansen-Dürr, P., A. Meichle, P. Steiner, M. Pagano, K. Finke, J. Botz, J. Wessbecher, G. Draetta, and M. Eilers.** 1993. Differential modulation of cyclin gene expression by *MYC*. Proc. Natl. Acad. Sci. USA **90**:3685–3689.
33. **Jiang, W., S. M. Kahn, P. Zhou, Y.-J. Zhang, A. M. Cacace, A. S. Infante, S. Doi, R. M. Santella, and I. B. Weinstein.** 1993. Overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth control, cell cycle progression and gene expression. Oncogene **8**:3447–3457.
34. **Karn, J., J. V. Watson, A. D. Lowe, S. M. Green, and W. Vedeckis.** 1989. Regulation of cell cycle duration by c-myc levels. Oncogene **4**:773–787.
35. **Kato, G. J., J. Barrett, M. Villa-Garcia, and C. V. Dang.** 1990. An amino-terminal c-myc domain required for neoplastic transformation activates transcription. Mol. Cell. Biol. **10**:5914–5920.
36. **Kato, J.-Y., H. Matsushime, S. W. Hiebert, M. E. Ewen, and C. J. Sherr.** 1993. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev. **7**:331–342.
37. **Katz, S., E. Kowenz-Leutz, C. Müller, K. Meese, S. A. Ness, and A. Leutz.** 1993. The NF-M transcription factor is related to C/EBP b and plays a role in signal transduction, differentiation and leukemogenesis of avian myelomonocytic cells. EMBO J. **12**:1321–1332.
38. **Keath, E. J., P. G. Caimi, and M. D. Cole.** 1984. Fibroblast lines expressing activated c-myc oncogenes are tumorigenic in nude mice and syngeneic animals. Cell **39**:339–348.
39. **Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder.** 1983. Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell **35**:603–610.
40. **Kirschmeier, P. T., G. M. Housey, M. D. Johnson, A. S. Perkins, and B. Weinstein.** 1988. Construction and characterization of a retroviral vector demonstrating efficient expression of cloned cDNA sequences. DNA **7**:219–225.
41. **Kirschner, M.** 1992. The cell cycle then and now. Trends Biochem. Sci. **17**:281–285.

41a. **Kowentz-Leutz, E., A. Leutz, and M. Eilers.** Unpublished data.

42. **Kretzner, L., E. M. Blackwood, and R. N. Eisenmann.** 1992. Myc

and Max proteins possess distinct transcriptional activities. Nature (London) **359:**426–429.
43. **Lanahan, A., J. B. Williams, L. K. Sanders, and D. Nathans.** 1992. Growth factor-induced delayed early response genes. Mol. Cell. Biol. **12:**3919–3929.
43a. **Li, L., C. Nerlov, G. Prendergast, D. MacGregor, and E. Ziff.** Personal communication.
44. **Lovec, H., A. Sewing, F. C. Lucibello, R. Müller, and T. Möröy.** 1994. Oncogenic activity of cyclin D1 revealed through cooperation with Ha-*ras*: link between cell cycle control and malignant transformation. Oncogene **9:**323–326.
45. **Lucibello, F. C., A. Sewing, S. Brusselbach, C. Burger, and R. Muller.** 1993. Induction of cyclin D1 and cyclin-E and suppression of cdk2 and cdk4 in senescent human cells. J. Cell Sci. **105:**123–133.
45a. **Lukas, J., M. Pagano, Z. Staskova, G. Draetta, and J. Bartek.** 1994. Cyclin D1 protein oscillates and is essential for cell cycle progression in human tumour lines. Oncogene **9:**404–418.
46. **Mäkelä, T. P., P. J. Koskinen, I. Väsrik, and K. Alitalo.** 1992. Alternative forms of Max as enhancers or suppressors of Myc-Ras cotransformation. Science **256:**373–376.
47. **Markowitz, D., S. Goff, and A. Bank.** 1988. A safe packaging line for gene transfer: separating viral genes on two different plasmids. J. Virol. **62:**1120–1124.
48. **Matsushime, H., M. F. Roussel, R. A. Ashmun, and C. J. Sherr.** 1991. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell **65:**701–713.
49. **Meichle, A., A. Philipp, and M. Eilers.** 1992. The functions of Myc proteins. Biochim. Biophys. Acta **1114:**129–146.
50. **Moses, H. L., E. Y. Yang, and J. A. Pietenpol.** 1990. TGF-beta stimulation and inhibition of cell proliferation: new mechanistic insights. Cell **63:**245–247.
51. **Motokura, T., T. Bloom, H. G. Kim, H. Jüppner, J. V. Ruderman, H. M. Kronenberg, and A. Arnold.** 1991. A novel cyclin encoded by a *bcl1*-linked candidate oncogene. Nature (London) **350:**512–515.
52. **Müller, H., J. Lukas, A. Schneider, P. Warthoe, J. Bartek, M. Eilers, and M. Strauss.** Cyclin D1 expression is regulated by the retinoblastoma protein. Proc. Natl. Acad. Sci. USA, in press.
53. **Nordeen, S. K.** 1988. Luciferase reporter gene vectors for analysis of promoters and enhancers. BioTechniques **6:**454–457.
53a. **Pagano, M., et al.** Personal communication.
54. **Pagano, M., M. Dürst, S. Joswig, G. Draetta, and P. Jansen-Dürr.** 1992. Binding of the human E2F transcription factor to the retinoblastoma protein but not to cyclin A is abolished in HPV-16-immortalized cells. Oncogene **7:**1681–1686.
55. **Penn, L. J. Z., M. W. Brooks, E. M. Laufer, and H. Land.** 1990. Negative autoregulation of c-myc transcription. EMBO J. **9:**113–121.
56. **Persson, H., and P. Leder.** 1984. Nuclear localization and DNA binding properties of a protein expressed by human c-myc oncogene. Science **225:**718–721.
56a. **Philipp, A., and M. Eilers.** Unpublished data.
57. **Pines, J., and T. Hunter.** 1992. Cyclins A and B1 in the human cell cycle. CIBA Found. Symp. **170:**187–196.
58. **Prendergast, G. C., R. Hopewell, B. J. Gorham, and E. B. Ziff.** 1992. Biphasic effect of Max on Myc cotransformation activity and dependence on amino- and carboxy-terminal Max functions. Genes Dev. **6:**2429–2439.
59. **Prendergast, G. C., D. Lawe, and E. B. Ziff.** 1991. Association of myn, the murine homolog of max, with c-myc stimulates methylation-sensitive DNA binding and ras cotransformation. Cell **65:** 395–407.
60. **Prendergast, G. C., and E. B. Ziff.** 1991. Methylation-sensitive sequence-specific DNA binding by the c-myc basic region. Science **251:**186–189.
61. **Quelle, D. E., R. A. Ashmun, S. A. Shurtleff, J. Y. Kato, S. D. Bar, M. F. Roussel, and C. J. Sherr.** 1993. Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. Genes Dev. **7:**1559–1571.
62. **Rosenwald, I. B., A. Lazaris-Karatzas, N. Sonenberg, and E. V. Schmidt.** 1993. Elevated levels of cyclin D1 protein in response to increased expression of eukaryotic initiation factor 4E. Mol. Cell. Biol. **13:**7358–7363.
63. **Roy, A. L., S. Malik, M. Meisterernst, and R. G. Roeder.** 1993. An alternative pathway for transcription initiation involving TfII-I. Nature (London) **365:**355–361.
64. **Roy, A. L., M. Meisterernst, P. Pognonec, and R. G. Roeder.** 1991. Cooperative interaction of an initiator-binding transcription initiation factor and the helix-loop-helix activator USF. Nature (London) **354:**245–248.
65. **Royer-Pokora, B., H. Beug, M. Claviez, H.-J. Winkhardt, R. R. Friis, and T. Graf.** 1978. Transformation parameters in chicken fibroblasts transformed by AEV and MC29 avian leukemia viruses. Cell **13:**751–760.
66. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
67. **Sarid, J., T. D. Halazonetis, W. Murphy, and P. Leder.** 1987. Evolutionarily conserved regions of the human c-myc protein can be uncoupled from transforming activity. Proc. Natl. Acad. Sci. USA **84:**170–173.
68. **Sawadogo, M., and R. G. Roeder.** 1987. DNA-binding specificity of USF, a human gene-specific transcription factor required for maximum expression of the major late promoter of adenovirus. Cancer Cells **4:**147–154.
68a. **Schneider, A., and M. Eilers.** Unpublished data.
68b. **Schneider, A., A. Roy, M. Eilers, and R. G. Roeder.** Unpublished data.
69. **Seto, E., Y. Shi, and T. Shenk.** 1991. YY1 is an initiator sequence-binding protein that directs and activates transcription *in vitro*. Nature (London) **354:**241–245.
70. **Sherr, C. J.** 1993. Mammalian $G_1$ cyclins. Cell **73:**1059–1065.
71. **Shrivastava, A., S. Saleque, G. V. Kalpana, S. Artandi, S. P. Goff, and K. Calame.** 1993. Inhibition of transcriptional regulator Yin-Yang-1 by association with c-Myc. Science **262:**1889–1891.
72. **Shtivelman, E., and J. M. Bishop.** 1991. Expression of *CD44* is repressed in neuroblastoma cells. Mol. Cell. Biol. **11:**5446–5453.
73. **Spencer, C. A., and M. Groudine.** 1991. Control of c-*myc* regulation in normal and neoplastic cells. Adv. Cancer Res. **56:**1–48.
74. **Spitkovsky, D., P. Steiner, J. Lukas, E. Lees, M. Pagano, A. Schulze, S. Joswig, D. Picard, M. Tommasino, M. Eilers, and P. Jansen-Dürr.** 1994. Modulation of cyclin gene expression by adenovirus E1A in a cell line with E1A-dependent conditional proliferation. J. Virol. **68:**2206–2214.
74a. **Steiner, P., and M. Eilers.** Unpublished data.
75. **Stone, J., T. de Lange, G. Ramsay, E. Jakobovits, J. M. Bishop, H. Varmus, and B. Lee.** 1987. Definition of regions in human c-*myc* that are involved in transformation and nuclear localization. Mol. Cell. Biol. **7:**1697–1709.
76. **Suen, T.-C., and M.-C. Hung.** 1991. c-*myc* reverses *neu*-induced transformed morphology by transcriptional repression. Mol. Cell. Biol. **11:**354–362.
77. **Sutherland, J. A., A. Cook, A. J. Bannister, and T. Kouzarides.** 1992. Conserved motifs in Fos and Jun define a new class of activation domain. Genes Dev. **6:**1810–1819.
78. **Wagner, A. J., C. Meyers, L. A. Laimins, and N. Hay.** 1993. C-myc induces the expression and activity of ornithine decarboxylase. Cell Growth Differ. **4:**879–883.
79. **Watson, D. K., E. P. Reddy, P. H. Duesberg, and T. S. Papas.** 1983. Nucleotide sequence analysis of the chicken c-*myc* gene reveals homologous and unique coding regions by comparison with the transforming gene of avian myelocytomatosis virus MC29, D*gag-myc*. Proc. Natl. Acad. Sci. USA **80:**2146–2150.
80. **Westin, E. H., R. C. Gallo, S. K. Arya, A. Eva, L. M. Souza, M. A. Baluda, S. A. Aaronson, and F. Wong-Staal.** 1982. Differential expression of the *amv* gene in human hematopoietic cells. Proc. Natl. Acad. Sci. USA **79:**2194–2198.
81. **Westin, E. H., F. Wong-Staal, E. P. Gelmann, R. Dalla-Favera, T. S. Papas, J. A. Lautenberger, A. Eva, E. P. Reddy, S. R. Tronik, S. A. Aaronson, and R. C. Gallo.** 1982. Expression of cellular homologues of retroviral *onc* genes in human hematopoietic cells. Proc. Natl. Acad. Sci. USA **79:**2490–2494.
82. **Won, K.-A., Y. Xiong, D. Beach, and M. Z. Gilman.** 1992. Growth-regulated expression of D-type cyclin genes in human diploid fibroblasts. Proc. Natl. Acad. Sci. USA **89:**9910–9914.
83. **Yang, B.-S., T. J. Geddes, R. J. Pogulis, B. de Crombrugghe, and S. O. Freytag.** 1991. Transcriptional suppression of cellular gene expression by c-Myc. Mol. Cell. Biol. **11:**2291–2295.