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The Myc Transactivation Domain Promotes Global Phosphorylation of the RNA Polymerase II Carboxy-Terminal Domain Independently of Direct DNA Binding▽†

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Myc is a transcription factor which is dependent on its DNA binding domain for transcriptional regulation of target genes. Here, we report the surprising finding that Myc mutants devoid of direct DNA binding activity and Myc target gene regulation can rescue a substantial fraction of the growth defect in *myc*$^{-/-}$ fibroblasts. Expression of the Myc transactivation domain alone induces a transcription-independent elevation of the RNA polymerase II (Pol II) C-terminal domain (CTD) kinases cyclin-dependent kinase 7 (CDK7) and CDK9 and a global increase in CTD phosphorylation. The Myc transactivation domain binds to the transcription initiation sites of these promoters and stimulates TFIIH binding in an MBII-dependent manner. Expression of the Myc transactivation domain increases CDK mRNA cap methylation, polysome loading, and the rate of translation. We find that some traditional Myc transcriptional target genes are also regulated by this Myc-driven translation mechanism. We propose that Myc transactivation domain-driven RNA Pol II CTD phosphorylation has broad effects on both transcription and mRNA metabolism.

Members of the Myc family are champion oncogenes (12, 45). N-Myc or c-Myc expression is frequently elevated in cancers of diverse origins, and transgenic mice with elevated Myc expression develop tumors in many organs. In normal cellular physiology, Myc induction is an integrator of extracellular signals which drive cell proliferation. Growth factors induce Myc expression, and this induction is necessary for cell proliferation (4, 19). During oncogenesis, Myc expression becomes elevated independently of growth factors and survival signals, permitting unrestrained cell proliferation.

Myc forms a heterodimer with Max and operates as a transcription factor which both activates and represses genes. Numerous microarray analyses have revealed that Myc is a weak but pleiotropic transcription factor, activating or repressing around 5 to 10% of all genes about 1.5- to 2-fold (16). A number of Myc cofactors facilitate this role. Myc binds to the TRRAP complex, recruiting histone acetylation activity to promoters, which promotes transcription by opening up chromatin structure (34, 35). Myc has also been found to bind and act in conjunction with other transcriptional regulators (15). In addition to driving transactivation, Myc has also been found to drive transcription elongation via the recruitment of P-TEFb (positive transcription elongation factor b) (18). Full Myc transcriptional activity is associated with rapid turnover of the protein, and Skp2, HectH9, and Fbw7 mediate this process (1, 24, 56–58). Regardless of the cofactor involved, transactivation ultimately must increase RNA polymerase II (Pol II) activity, but exactly how Myc does this remains unclear.

Myc/Max was first characterized as a sequence-specific transcription factor that binds to a consensus site, CACGTG (6). This sequence has been validated as the binding site required for stimulated transactivation of many target genes (http://www.myc-cancer-gene.org). However, the necessity for Myc to function by binding to the Myc consensus sequence has been called into question by in vivo Myc binding studies which have placed Myc at promoters without consensus binding sites (10, 20, 26, 30, 44). Currently, it remains unclear whether Myc functions at all of these nonconsensus sites and indeed whether there is always a consequence to Myc binding near a promoter. Myc has not been found to regulate transcription at many of the promoters it binds to, but the possibility remains that Myc has some transcription-independent role at these sites.

RNA polymerase II activity is governed by a cycle of phosphorylation and dephosphorylation of the large subunit C-terminal domain (CTD) (5, 29, 52). Hypophosphorylated RNA Pol II is recruited to initiation complexes, and transcription initiation is associated with TFIIH-driven CTD serine-5 (S5) phosphorylation (42). After 20 to 50 nucleotides have been transcribed, RNA becomes capped by the addition of an inverted 7-methylguanosine triphosphate in a series of reactions which are most efficient when cotranscriptional (13, 46). Capping requires three sequential enzymatic activities. RNA triphosphatase removes the terminal phosphate from the first nucleotide, and guanylyltransferase adds an inverted guanylyl to the mRNA. In mammals, both of these enzymes are present in the same polypeptide, which is recruited and activated by the S5-P CTD (11, 33, 55, 59). The third step is 7-methylation of the guanosine, and the methyltransferase is also recruited by the S5-P CTD (25, 48). Guanosine addition stabilizes the mRNA against degradation, but the mRNA becomes competent to be translated only when methylated (50, 51). Since the capping enzymes are recruited and activated by the phosphorylated CTD, transcription and mRNA processing are intricately linked and codependent.

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We initiated a study of Myc biological activity that is independent of direct DNA binding and transcriptional activation or repression. We present the striking finding that Myc can promote a global elevation in phosphorylation of the RNA Pol II CTD which has a potentially broad impact on transcription, mRNA processing, and translation.

MATERIALS AND METHODS

Cell culture, transfection, retroviral infection, siRNA transfection, and assays. Rat-1fibroblasts (TGR cells), rat c-*myc* null fibroblasts (HO15.19 cells), Tet-21/N cells, and retroviral producer PhoeNX cells were cultured in Dulbecco modified Eagle medium-10% fetal calf serum. Immortalized mammary epithelial cells (IMECs) were cultured as previously described (17). Retroviral infection of HO15.19 and IMECs was performed using PhoeNX cells. Tet-21/N cells were maintained in 0.2 μg/ml doxycycline for 2 days to repress N-*myc* expression. For growth curves, 10^4 cells from log-phase cultures were plated in six-well plates. Every 24 h, cells were trypsinized and counted using a hemocytometer. Cells expressing cyclin-dependent kinase 7 (CDK7) dominant negative (DN) or the vector control were counted 2 days following infection. A 10-cm plate of TGR cells was transfected with 24 μl 1.2 nM small interfering RNA (siRNA; Dharmacon) (scrambled control, Myc siRNA#1 or Myc siRNA#2; sequences available upon request). After 24 h, cells were processed as appropriate.

Microarrays. RNA was harvested from two independent cultures of *myc* null rat fibroblasts (HO15.19) expressing the vector control, MycWT, MycΔMBII, and MycBM using an RNeasy kit (QIAGEN). RNA integrity and concentration were verified by gel electrophoresis. Arrays were performed as described previously (14). Only genes with a signal >20% above background and with at least 70% good data across the arrays were considered for further analysis. The data were centered to the average of the two vector-only arrays and filtered for average changes in signal for either MycWT or mutants.

Immunoprecipitations. To detect Myc/Max interactions in $myc^{-/-}$ cells, a 15-cm plate of cells was lysed in 1 ml F buffer (53) and extracts incubated with anti-FLAG antibody-conjugated beads (Sigma) for 3 h at 4°C. Immunoblot analyses were performed on immunoprecipitated protein using anti-N-Myc or anti-Max polyclonal antibody (Santa Cruz). To detect Myc/CDK7 interactions in 293 cells, a 10-cm plate of 293 cells was transfected with 1 μg expression vector for N-MycWT or N-MycΔMBII and 1 μg expression vector for CDK7 or the relevant vector control. After 2 days, nuclei were prepared by hypotonic lysis and extracted with Dignam C buffer. Extracts were mixed 1:1 with F buffer, and 25% of each extract was used for an immunoprecipitation (IP) using polyclonal anti-CDK7 or anti-N-Myc antibody (Santa Cruz). Immunoblot analyses were performed on immunoprecipitated protein using monoclonal anti-CDK7 (Santa Cruz) or N-myc (Upstate) antibody. For the CDK-activating kinase (CAK) assay, CDK7 kinase activity was measured as described previously (32) using recombinant CDK2 (Santa Cruz) as a substrate. To detect endogenous Myc/CDK7 interactions in 293 cells, nuclei from 4- by 15-cm plates were extracted with Dignam C buffer. Cell extracts were mixed 1:1 with F buffer and precleared for 1 h using anti-mouse antibodies. The nuclear extract was divided, and immunoprecipitation was carried out with either anti-c-Myc antibodies (C33; Santa Cruz) or control monoclonal antibodies. Western blot analyses were performed as described above.

Chromatin immunoprecipitation. The Upstate chromatin immunoprecipitation (ChIP) assay kit was used. IPs were carried out using polyclonal anti-FLAG antibody (Sigma) and polyclonal antibodies raised against N-Myc, MAT1, p62, or control antibody (Santa Cruz). PCR was carried out on the resultant DNA samples and 1% of the input using ^{32}P end-labeled primer pairs (sequences available upon request). PCR products were resolved on 5% Tris-borate-EDTA–polyacrylamide gel electrophoresis (PAGE), visualized by a phosphorimager, and quantitated using ImageQuant software. For each reaction, at least three independent ChIPs were performed and the PCRs were monitored to be well within the linear range.

RT-PCR. RNA was extracted from log-phase cells by using the RNeasy kit (QIAGEN) and normalized. Reverse transcription (RT)-PCR was carried out using the Platinum quantitative RT-PCR system (Invitrogen). The annealing temperature was 55°C, and the number of cycles to meet the linear range of the reaction for all primers pairs was determined (typically between 18 and 22). The primer sequences are available upon request. Products were quantitated as described above. The mean expression for two independent RNA samples was calculated, with error bars indicating standard deviations.

Polysome preparation. Polysomes were prepared from log-phase cells by cell extract centrifugation over 30% sucrose as described previously (9). RNA was extracted using TRIzol from the monosome/hnRNA fraction at the sucrose buffer interface and from the polysome pellet. The distribution of specific mRNAs in each fraction was determined by performing RT-PCR.

Anti-methyl cap IP. RNA was extracted by TRIzol followed by phenol-chloroform, and 2 μg was suspended in 100 μl binding buffer (25 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mg/ml tRNA [baker's yeast; Roche], 0.1 mg/ml bovine serum albumin). Anti-2,2,7-methylguanosine antibody-bound beads or antibody control-bound beads (Calbiochem) were blocked with binding buffer. RNA was rotated for 1 h at 4°C with 20 μl anti-2,2,7-methylguanosine antibody-bound beads or 40 μl antibody control-bound beads to control for nonspecific binding. Beads and flowthrough were separated, and the volume was increased to 200 μl by adding binding buffer. Samples were extracted with phenol-chloroform, precipitated with ethanol, and resuspended in 50 μl H_2O. Two microliters of RNA was used for each 20-μl RT-PCR volume, and RT-PCR was performed as described above except that 24 to 27 cycles were used.

Immunoblots. Two days following seeding, subconfluent cells were lysed in a modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 130 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 20 mM NaF, 1 μg/ml of leupeptin, pepstatin, and aprotinin, 1 mM dithiothreitol, and phosphatase inhibitor cocktails I and II [Sigma]). Protein concentration was determined using a modified Lowry protein assay kit (Pierce). Equivalent amounts of protein were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and immunoblotted using antibodies raised against the proteins indicated in the figures. To detect RNA Pol II, nuclei were isolated by Dounce homogenization of the cell pellet in Dignam A buffer. Nuclear proteins were extracted by resuspension in Dignam C or F buffer. Equivalent amounts of protein were immunoblotted for RNA Pol II (Santa Cruz) and anti-CTD phospho-S5 (H14) and anti-CTD phospho-S2 (H5) (Covance).

EMSA. An electrophoretic mobility shifty assay (EMSA) was performed as previously described (53). Complexes were identified by incubating 0.2 μg of the relevant polyclonal antibody with the reaction mixes to assay for supershift or loss of the band (not shown). Complexes were separated on a 5% acrylamide gel and visualized by a phosphorimager.

In vivo cell labeling experiments. For each IP, a 10-cm plate of $myc^{+/+}$ fibroblasts or $myc^{-/-}$ fibroblasts expressing MycWT, MycBM, MycΔC, or the vector control was incubated for 30 min in 4 ml Dulbecco modified Eagle medium without cysteine and methionine (Sigma) and in 10% dialyzed fetal calf serum. Promix (10 μl; Amersham) was added for 0.5, 1, or 1.5 h as indicated. Chase was performed by washing the cells three times and incubating them in normal growth medium for 2, 4, or 6 h as indicated. RIPA extracts were made and normalized for protein content. Samples were precleared by 1 h of incubation with 25 μl normal rabbit immunoglobulin G agarose. Polyclonal cyclin T1 or CDK9 antibody (1 μg) was incubated with the extracts overnight, and then 25 μl protein A/G-Sepharose was added for 1 h. Beads were washed in RIPA buffer and eluted in sample buffer. Immunoprecipitated protein and cell extracts were resolved on 8% SDS-PAGE. Bands were visualized using a Storm phosphorimager and quantitated using ImageQuant software. Each experiment was performed on at least two independent occasions. The experiment whose results are shown is representative, and error bars indicate the standard deviations for duplicates. Total label incorporation was calculated by trichloroacetic acid (TCA) precipitation of cell extracts, followed by label detection using a scintillation counter.

RESULTS

Design and characterization of Myc mutants defective for DNA binding. We are interested in the biological activity of the Myc transactivation domain and wanted to separate the function of this domain from the downstream effects of Myc target genes. Such analysis can reveal novel Myc activities as well as provide insight into wild-type Myc function. To this end, we created two mutants of murine N-Myc, called MycBM and MycΔC, which could not bind to DNA directly (Fig. 1A). In MycBM, the C-terminal, basic region which binds to the DNA phosphate backbone is mutated into an acidic region, i.e., amino acids 381 to 384, RQRR, are mutated to ADAA. MycΔC has a deletion of the entire DNA binding and Max dimerization domain (amino acids 372 to 454) and a nuclear

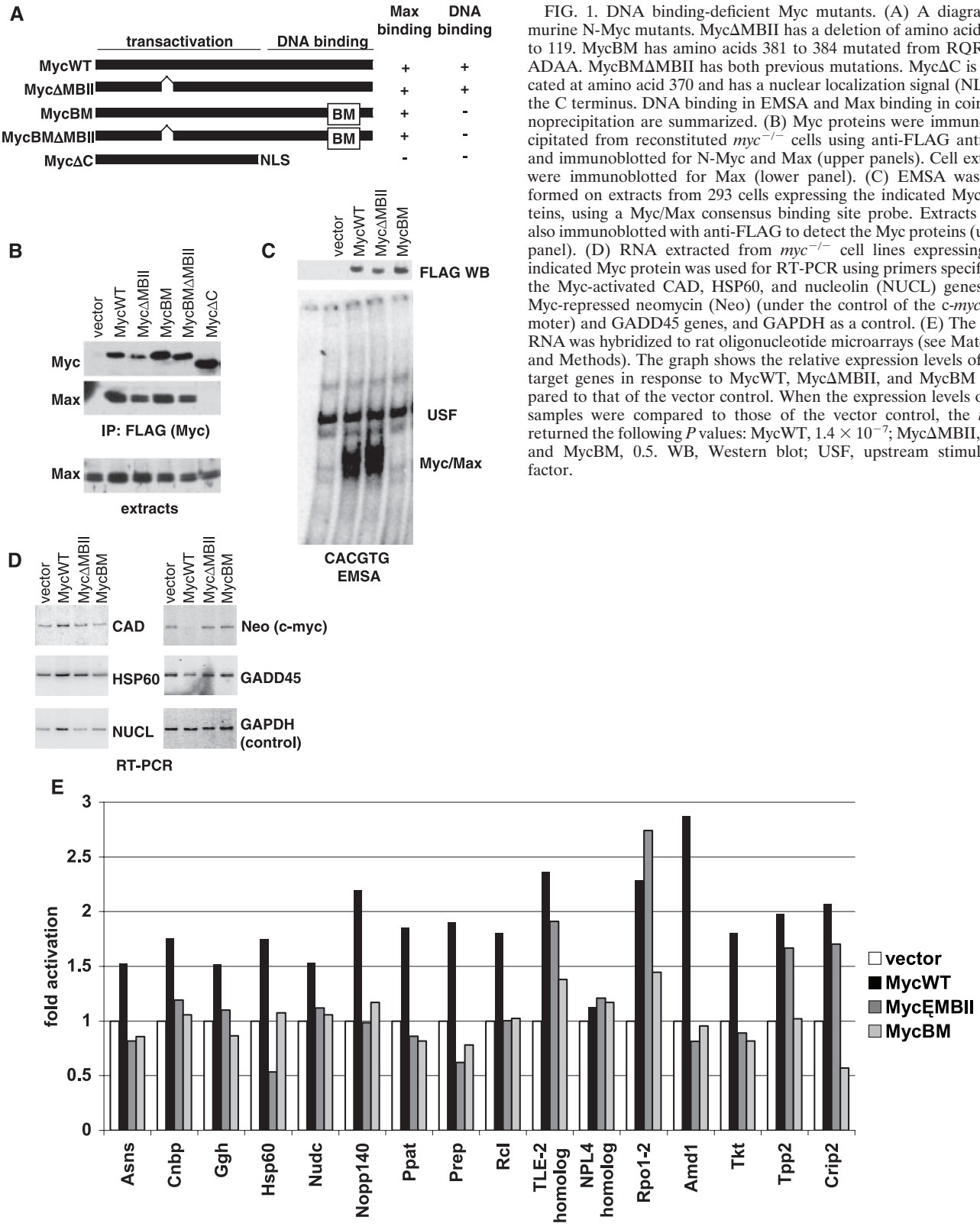

FIG. 1. DNA binding-deficient Myc mutants. (A) A diagram of murine N-Myc mutants. MycΔMBII has a deletion of amino acids 103 to 119. MycBM has amino acids 381 to 384 mutated from RQRR to ADAA. MycBMΔMBII has both previous mutations. MycΔC is truncated at amino acid 370 and has a nuclear localization signal (NLS) at the C terminus. DNA binding in EMSA and Max binding in coimmunoprecipitation are summarized. (B) Myc proteins were immunoprecipitated from reconstituted $myc^{-/-}$ cells using anti-FLAG antibody and immunoblotted for N-Myc and Max (upper panels). Cell extracts were immunoblotted for Max (lower panel). (C) EMSA was performed on extracts from 293 cells expressing the indicated Myc proteins, using a Myc/Max consensus binding site probe. Extracts were also immunoblotted with anti-FLAG to detect the Myc proteins (upper panel). (D) RNA extracted from $myc^{-/-}$ cell lines expressing the indicated Myc protein was used for RT-PCR using primers specific for the Myc-activated CAD, HSP60, and nucleolin (NUCL) genes, the Myc-repressed neomycin (Neo) (under the control of the c-*myc* promoter) and GADD45 genes, and GAPDH as a control. (E) The same RNA was hybridized to rat oligonucleotide microarrays (see Materials and Methods). The graph shows the relative expression levels of Myc target genes in response to MycWT, MycΔMBII, and MycBM compared to that of the vector control. When the expression levels of the samples were compared to those of the vector control, the *t* test returned the following *P* values: MycWT, 1.4×10^{-7}; MycΔMBII, 0.09; and MycBM, 0.5. WB, Western blot; USF, upstream stimulatory factor.

localization signal added at the C terminus. These two DNA binding mutants were designed to be complementary. MycBM retains the Max binding domain whereas MycΔC does not. Equivalent cellular responses to the expression of both mutants are unlikely to be due to Max titration. To further investigate the role of the N terminus of Myc, an additional deletion of amino acids 103 to 119, encompassing the Myc homology box II domain (MBII), was made in the full-length Myc and MycBM proteins, creating MycΔMBII and MycBMΔMBII, respectively. MBII is highly conserved in evolution and is necessary for nearly all reported N-terminal interactions and functions, including oncogenic transformation (54).

We confirmed that MycBM and MycΔC did not bind to the Myc consensus sites or regulate Myc target genes and that they bound to Max as predicted. To assess Max interaction, Myc proteins were immunoprecipitated via their FLAG tag and immunoblotted for Myc and Max (Fig. 1B). As predicted, MycWT, MycBM, and their respective ΔMBII mutants were able to bind to endogenous Max, whereas MycΔC could not. Since MycBM retains much of the DNA binding domain, we wanted to confirm that it could not bind to Myc consensus sites. An EMSA was performed using transiently expressed proteins and the Myc/Max consensus binding site, CACGTG, as a probe. MycWT, MycΔMBII, and MycBM were expressed at equivalent levels (Fig. 1C, upper panel). In the EMSA, Myc/Max and MycΔMBII/Max heterodimers could bind to a CACGTG probe and were detected as a characteristic band that migrated faster than the endogenous upstream stimulatory factor-DNA complex. MycBM did not have detectable DNA binding activity, despite the fact that it dimerized avidly with Max (Fig. 1B). The presence of both Myc and Max in the DNA binding complexes was confirmed by a supershift using anti-Max and anti-Myc antibodies (data not shown).

To confirm that MycBM was not regulating Myc target genes, the transcription of many established Myc-activated and -repressed genes was measured (Fig. 1D and E). Quantitative RT-PCR was performed on RNA extracted from pools of *myc* null cells expressing MycWT, MycΔMBII, and MycBM. MycWT was found to increase transcription of the established Myc-activated CAD, HSP60, and nucleolin genes two- to threefold, but MycBM did not increase the transcription of these genes above background levels (Fig. 1D). Transcription of established Myc-repressed genes, the GADD45 and endogenous c-*myc* (reported by the neomycin gene driven by the c-*myc* promoter in *myc* null cells) genes, were repressed about fivefold by MycWT, but MycBM failed to repress either target. Microarray analysis concurred that MycBM does not activate Myc target genes. Previously, Myc target genes in the rat fibroblast system had been established using microarrays and auxiliary techniques (43). We found 21 previously reported Myc target genes to be upregulated by the expression of MycWT in our experiments (Fig. 1E). In contrast, the expression of MycBM does not upregulate any of these genes, and the vector control values and MycBM values are not significantly different, giving a P value of 0.4 using the t test. An equivalent microarray analysis of the transcriptional response to MycΔC was carried out and showed that MycΔC also does not upregulate Myc target genes (not shown).

Recent findings have shown that Myc is a regulator of rRNA synthesis, and it is hypothesized that this has a role in mediating the Myc growth phenotype (3, 21, 22). We found that the expression of MycWT increased total RNA content per cell (the majority of which is rRNA) about 1.5-fold above that of vector control cells (not shown). As predicted from the observation that MycBM cannot activate target genes, the expression of MycBM did not elevate cellular RNA content.

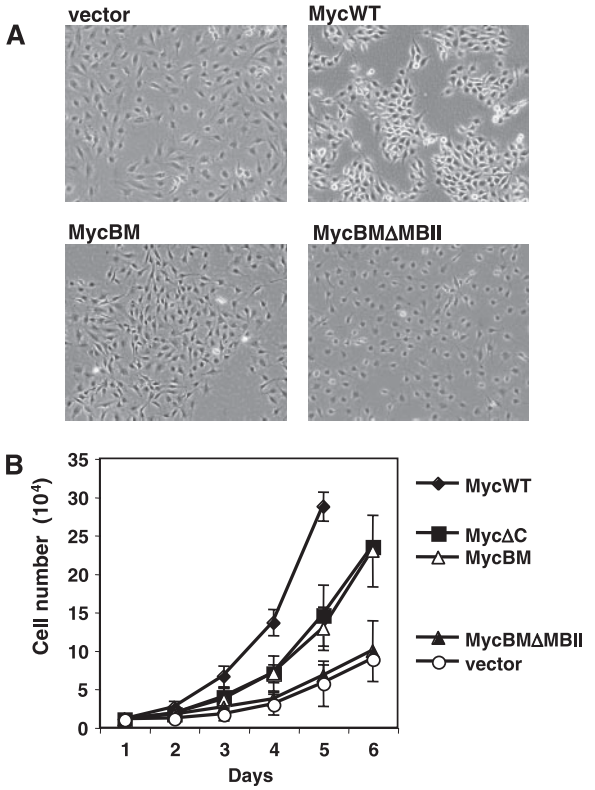

FIG. 2. DNA binding-deficient Myc mutants induce cell proliferation and morphological change. The results of an analysis of $myc^{-/-}$ cells expressing MycWT and mutants are shown. (A) Phase-contrast micrographs of log-phase cells. (B) Growth curves. The data shown represent the means for at least three experiments, and error bars show the standard deviations.

Myc induces morphological changes independently of target gene expression. To investigate the activity of the Myc N terminus independent of target genes, MycWT and the DNA binding mutants were stably expressed in the *myc* null rat fibroblast cell line HO15.19 (31). The $myc^{-/-}$ background allows Myc mutant phenotypes to be revealed rather than masked by endogenous Myc. Equivalent expression levels of the Myc proteins were verified by immunoblotting (Fig. 1B). $myc^{-/-}$ fibroblasts are broad, flat, and nonrefractile. As previously reported, the expression of MycWT reverted the $myc^{-/-}$ cell morphology to become comparable to that of Rat1 fibroblasts, the $myc^{+/+}$ line from which the $myc^{-/-}$ cells were derived (31). The MycWT-reconstituted cells appear smaller (although their volume is unchanged), darker, and refractile (Fig. 2A). MycWT cells also grew to a higher density than the vector control $myc^{-/-}$ cells (not shown). Surprisingly, the expression of MycBM partially rescued the morphology of $myc^{-/-}$ cells. MycBM cells appeared darker, smaller, and more refractile than vector control cells (Fig. 2A). They were also

able to grow to a higher density than vector cells (not shown). This phenotype was immediately apparent upon the expansion of polyclonal cell populations and reproduced in three independent infections. The morphological changes driven by MycBM were completely dependent on an intact N terminus because MycBMΔMBII cells had the same morphology as vector control cells (Fig. 2A). Therefore, the N terminus of Myc can partially rescue $myc^{-/-}$ cell morphology independently of direct DNA binding and target gene expression.

The proliferation defect in $myc^{-/-}$ cells can be partially rescued by Myc DNA binding mutants. Cell proliferation is dependent on Myc expression, and therefore, we investigated whether MycBM and MycΔC could rescue the proliferation defect of $myc^{-/-}$ cells. The doubling times of the cell lines were measured by counting equivalently seeded cells on consecutive days (Fig. 2B). In line with previous publications, $myc^{-/-}$ cells had a doubling time of 40 h and MycWT-expressing cells had a doubling time of 19 h (31). The expression of both MycBM and MycΔC resulted an intermediate doubling time of 28 h. Thus, a significant proportion of the growth defect in $myc^{-/-}$ cells can be restored by Myc proteins that cannot regulate target gene transcription. Consistent with morphological changes, this proliferation rescue was dependent on an intact N terminus, since the MycBMΔMBII cells had a doubling time of 36 h, close to that of the vector control. Moreover, this proliferative activity is independent of Max titration, since MycBM can bind to Max whereas MycΔC cannot. The ability of Myc DNA binding mutants to rescue the $myc^{-/-}$ cell proliferation defect was confirmed by cell cycle analysis using propidium iodide staining and fluorescence-activated cell sorter analysis (not shown). MycBM and MycΔC, unlike MycWT, do not rescue the growth defect in serum-starved cells and do not induce apoptosis or cell transformation (not shown).

Myc increases total cellular CAK activity. Having demonstrated a potent biological activity for the Myc N terminus, we were interested in resolving the mechanism of this activity and its relationship to wild-type Myc function. Myc-induced cell proliferation is correlated with an increase in cell cycle CDK activity (32). CDK complexes are activated by CAK, which phosphorylates the CDK T loop (23, 37). Since we had found that the expression of MycBM and MycΔC accelerated proliferation in a target gene-independent manner, we investigated whether total cellular CAK activity is increased in response to the expression of MycWT and mutants by using a CAK assay (Fig. 3A). The CAK assay only indicates the level of CAK activity in a cell line and does not distinguish increases in the specific activity of CAK from increases in the concentration of CAK itself, which are addressed below. CAK was immunoprecipitated from cell extracts by using an anti-CDK7 antibody and incubated with CDK2 as a substrate (Fig. 3A). In the representative assay whose results are shown, CAK activity in MycWT cell extracts was found to be 1.9-fold over that of the vector control, as reported previously (32). CAK activity was also elevated in MycBM cell extracts (1.7-fold over that of the vector control). Increased CAK activity was dependent on an intact N terminus because extracts from MycBMΔMBII cells have the same level of kinase activity as vector control cells (0.8-fold). In correlation with increased total cellular CAK activity, we found elevated levels of phospho-CDC2 and phospho-CDK2 in cells expressing MycWT, MycBM, and MycΔC compared to those in the vector control and MycBMΔMBII (not shown).

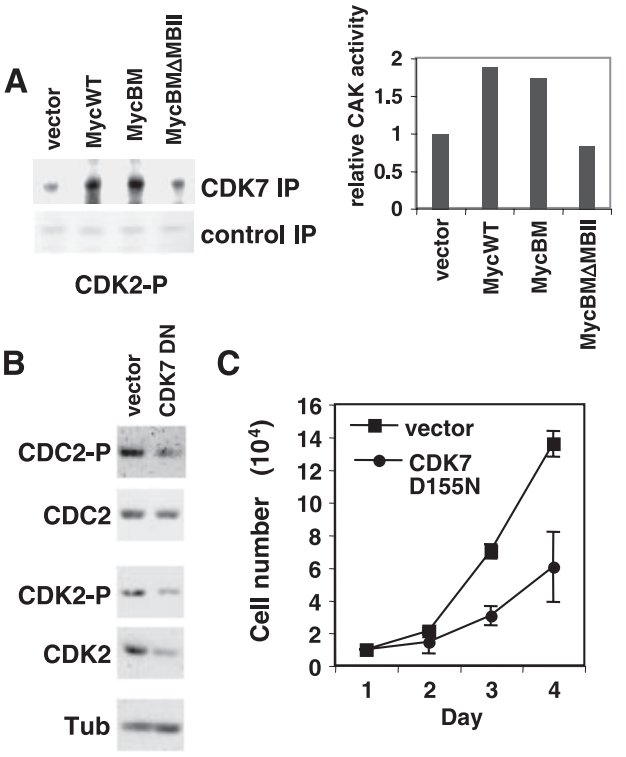

FIG. 3. The DNA binding-deficient Myc mutant elevates total cellular CAK activity. (A) A CAK assay was performed on extracts from $myc^{-/-}$ fibroblasts expressing the indicated Myc protein. Protein immunoprecipitated with anti-CDK7 or control antibody was incubated with recombinant CDK2 and [^{32}P]ATP, and reaction products were run on a gel, visualized by a phosphorimager (left panel), and quantitated (right panel). The results of a representative experiment are shown. (B) Cell extracts were prepared from $myc^{+/+}$ fibroblasts (TGR) expressing the vector control and CDK7 DN as indicated. Extracts were immunoblotted using antibodies raised against phospho-CDC2 (CDC2-P), CDC2, phospho-CDK2 (CDK2-P), CDK2, and γ-tubulin (Tub). (C) Growth curves for $myc^{+/+}$ fibroblasts (TGR) expressing the vector control and CDK7 DN. The data shown represent the means for two experiments, and error bars show the standard deviations.

To demonstrate that CAK has a critical role in cell proliferation and T-loop phosphorylation in vivo, we expressed a catalytically inactive CDK7 mutant, CDK7D155N, in the $myc^{+/+}$ fibroblast cell line TGR (28). Expression of this dominant interfering CDK7 inhibited both CDC2 and CDK2 phosphorylation levels and CDK2 expression (Fig. 3B). The cell proliferation rate was also inhibited by CDK7D155N (Fig. 3C).

Myc increases CAK expression independently of Myc target genes. CAK is a complex of CDK7, cyclin H, and MAT1. We investigated whether the increased CAK activity found in Myc-expressing cells could be a result of increased CAK component protein expression (Fig. 4A). To investigate whether CAK component expression was upregulated in response to endogenous Myc expression, we compared the parental $myc^{+/+}$ fibroblasts with $myc^{-/-}$ fibroblasts and we used two independent Myc siRNAs to knock down endogenous Myc expression in $myc^{+/+}$ fibroblasts. Both cyclin H and MAT1 expression levels were re-

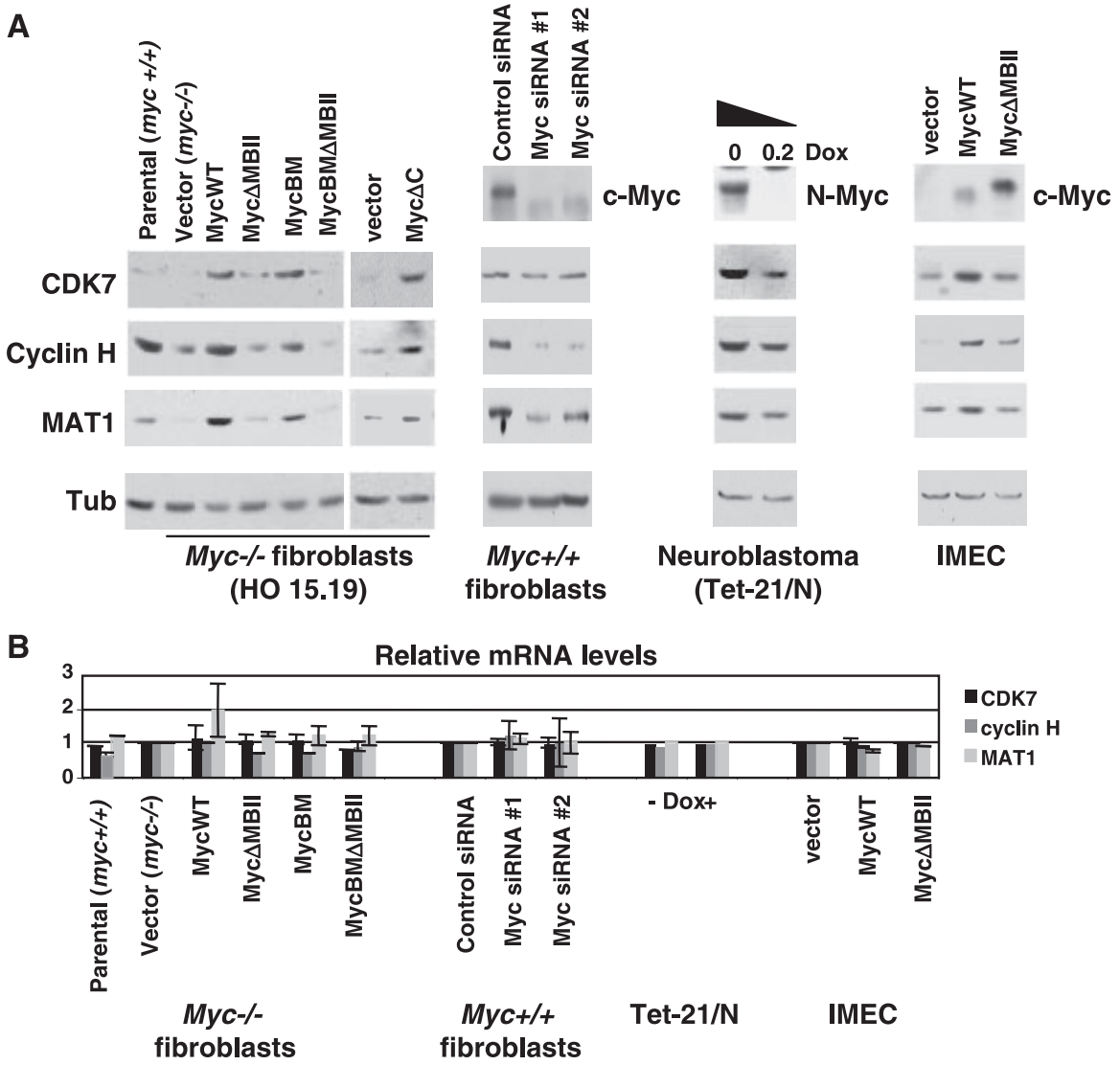

FIG. 4. Myc induces CAK expression by a posttranscriptional mechanism. (A) Cell extracts were prepared from $myc^{+/+}$ or $myc^{-/-}$ fibroblasts expressing the indicated Myc protein from several cell systems: $myc^{+/+}$ fibroblasts transfected for 24 h with control siRNA or two independent Myc siRNAs, a neuroblastoma cell line (Tet-21/N) expressing doxycycline-off N-Myc cultured in 0 and 0.2 μg/ml doxycycline (Dox) for 2 days, and IMECs expressing c-MycWT or c-MycΔMBII. Immunoblot analyses were performed on cell extracts using antibodies raised against CAK components, CDK7, cyclin H, and MAT1, and γ-tubulin (Tub). Immunoblot analyses were also performed using anti-c-Myc antibodies on $myc^{+/+}$ cell extracts and IMEC extracts and using anti-N-Myc antibodies on neuroblastoma cell extracts. (B) RNA was extracted from two independent log-phase samples. RT-PCR was performed in the linear range using primers specific for CDK7, cyclin H, MAT1, and GAPDH. Mean relative expression levels were calculated, and error bars indicate the standard deviations.

duced in $myc^{-/-}$ fibroblasts compared to those in $myc^{+/+}$ cells and $myc^{+/+}$ cells transfected with both Myc siRNAs but not the control siRNA. Next, we investigated CAK component expression in response to exogenous MycWT and the DNA binding mutants. In agreement with previous reports, we found a substantial increase in CDK7, cyclin H, and MAT1 protein expression in response to exogenous MycWT (32). Interestingly, we also found a comparable increase in CAK protein expression in cells expressing MycBM and MycΔC. These effects were dependent on an intact N terminus because cells expressing MycΔMBII and MycBMΔMBII did not express increased levels of CDK7, cyclin H, and MAT1. Thus, the increased cellular CAK activity in MycWT-, MycBM-, and MycΔC-expressing cells can be well correlated with elevated protein expression, and it is unlikely that the specific activity of CAK itself increases.

We wanted to determine how CAK components were being upregulated in response to Myc. CDK7 and MAT1 have never been reported as Myc target genes, and cyclin H is reported as a Myc-repressed gene (http://www.myc-cancer-gene.org) (49). However, we wanted to determine whether the transcription of these genes is activated in our specific cell system. Although the MycBM and MycΔC proteins lack DNA binding activity and activation of traditional Myc target genes, it was possible that they could upregulate the transcription of members of the CAK complex indirectly. We measured mRNA levels by a carefully controlled quantitative RT-PCR, carried out in the linear range on

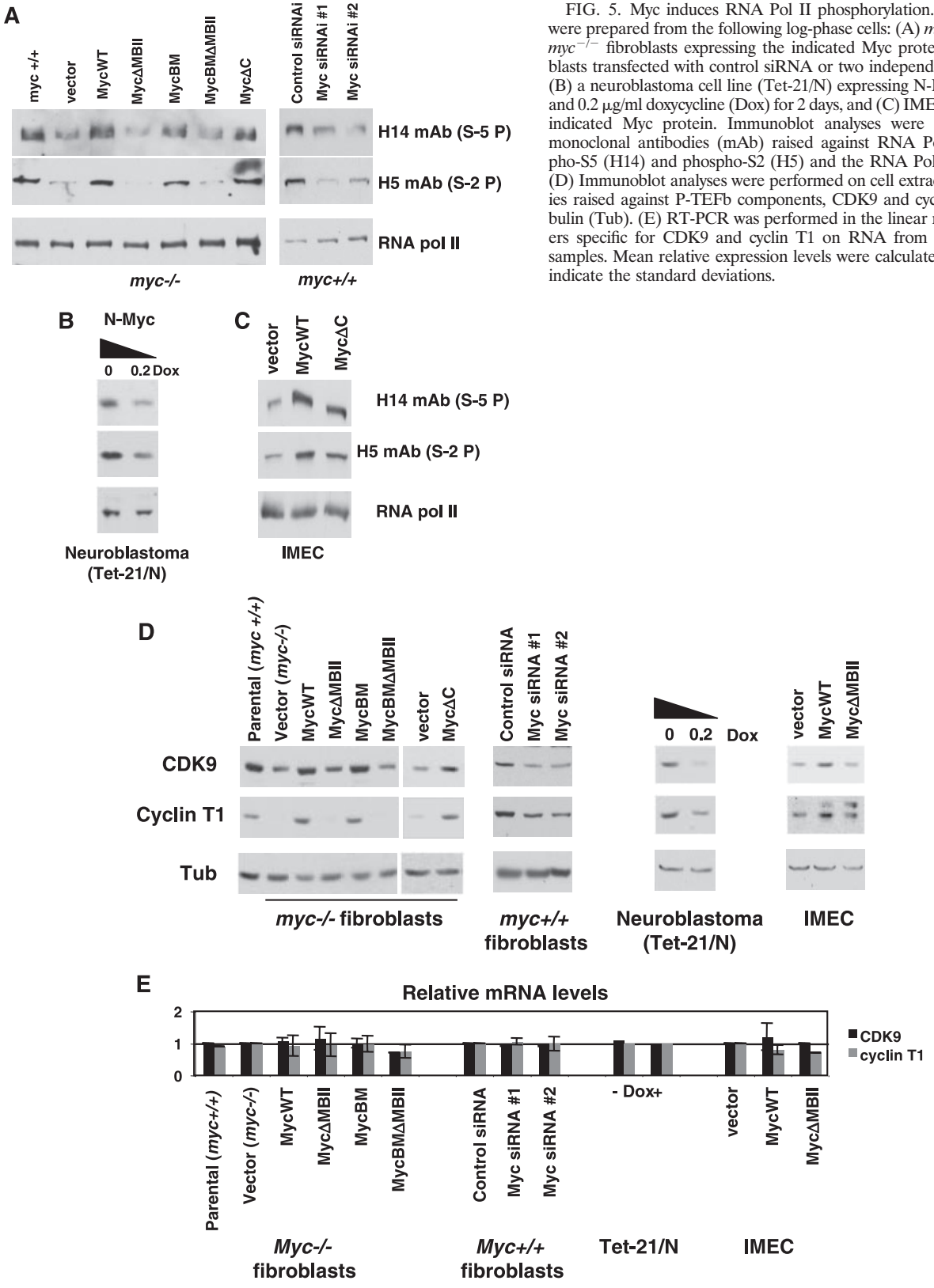

FIG. 5. Myc induces RNA Pol II phosphorylation. Nuclear extracts were prepared from the following log-phase cells: (A) $myc^{+/+}$ fibroblasts, $myc^{-/-}$ fibroblasts expressing the indicated Myc protein, $myc^{+/+}$ fibroblasts transfected with control siRNA or two independent Myc siRNAs, (B) a neuroblastoma cell line (Tet-21/N) expressing N-Myc cultured in 0 and 0.2 μg/ml doxycycline (Dox) for 2 days, and (C) IMECs expressing the indicated Myc protein. Immunoblot analyses were performed using monoclonal antibodies (mAb) raised against RNA Pol II CTD phospho-S5 (H14) and phospho-S2 (H5) and the RNA Pol II large subunit. (D) Immunoblot analyses were performed on cell extracts using antibodies raised against P-TEFb components, CDK9 and cyclin T1, and γ-tubulin (Tub). (E) RT-PCR was performed in the linear range using primers specific for CDK9 and cyclin T1 on RNA from two independent samples. Mean relative expression levels were calculated, and error bars indicate the standard deviations.

independent RNA samples. We found that CDK7, cyclin H, or MAT1 mRNA was not induced in response to modulation of endogenous Myc expression, both when comparing $myc^{+/+}$ with $myc^{-/-}$ fibroblasts and in response to both Myc siRNAs (Fig. 4B). We also looked at CAK component expression in response to exogenous MycWT and DNA binding mutants. We found that CDK7, cyclin H, or MAT1 mRNA was not induced in cells expressing MycBM or MycΔC compared to that in the vector control (Fig. 4B). These results were confirmed by microarray analysis (not shown). There was also no induction of CDK7 or cyclin H by MycWT. MAT1 mRNA was elevated in response to exogenous MycWT (and not in response to endogenous Myc levels). We conclude that endogenous Myc and the transcription-defective mutants MycBM and MycΔC lead to elevated CAK expression through a posttranscriptional mechanism.

We were interested in whether CAK protein expression was Myc responsive in other cell systems. We investigated CAK expression in cells from other lineages and in response to c-Myc as well as N-Myc. We examined CAK subunit expression in Tet-21/N cells, a neuroblastoma cell line that expresses N-Myc under doxycycline regulation (27), and in IMECs, a low-passage immortalized mammary epithelial line, engineered to stably express exogenous c-MycWT or c-MycΔMBII (17). We found increased expression of CDK7, cyclin H, and MAT1 proteins in response to elevated N-Myc expression in the neuroblastoma cell line and in response to elevated c-MycWT but not c-MycΔMBII in the IMECs (Fig. 4A). In agreement with the fibroblast data, Myc-driven upregulation of these proteins was found to be entirely posttranscriptional. CDK7, cyclin H, or MAT1 mRNA was not Myc regulated in either cell line (Fig. 4B). For the mammary epithelial cell line, this result was also confirmed by microarray analysis of RNA extracted from log-phase cells (not shown).

In summary, Myc can increase the expression of CAK proteins by a posttranscriptional mechanism in a variety of cell lines, in response to exogenous and endogenous Myc levels, and in response to both c-Myc and N-Myc.

Myc expression elevates cellular TFIIH and P-TEFb activity by a posttranscriptional mechanism. The CAK components CDK7, cyclin H, and MAT1 are also components of the basal transcription factor TFIIH. CDK7 is the TFIIH kinase which phosphorylates the RNA Pol II CTD S5 (52). Since we had found elevated CAK expression in response to Myc, we investigated whether this also led to increased TFIIH kinase activity. The protein expression level of RNA Pol II large subunit was unaltered in response to modulation of Myc expression in rat fibroblasts, the Tet-21/N neuroblastoma, and IMEC lines (Fig. 5, bottom panels). However, Myc expression was rate limiting for RNA Pol II CTD S5 phosphorylation, as detected by phospho-specific antibodies (Fig. 5A). S5 phosphorylation was reduced in $myc^{-/-}$ fibroblasts compared to that in $myc^{+/+}$ fibroblasts and was also reduced in $myc^{+/+}$ fibroblasts in which endogenous Myc was knocked down by two siRNAs. CTD S5 phosphorylation was also elevated in response to the expression of exogenous MycWT in fibroblasts, neuroblastoma cells, and epithelial cell lines (Fig. 5A to C). Furthermore, S5 phosphorylation was increased in response to the expression of the transcription-defective mutants MycBM and MycΔC but not MycBMΔMBII (Fig. 5A). Therefore, Myc-induced CTD S5 phosphorylation is dependent on the Myc N terminus alone but is also induced by MycWT. It is important to stress that the elevated CTD phosphorylation found in Myc-expressing cells is sufficiently large to be detected in the total nuclear pool of RNA Pol II. Thus, TFIIH is Myc regulated and rate limiting for CTD phosphorylation.

The S2 position of the RNA Pol II CTD is also phosphorylated, predominantly by P-TEFb. P-TEFb consists of CDK9 and several cyclins, most commonly cyclin T1. We found that Myc expression upregulates the subunits of P-TEFb (Fig. 5D). Endogenous Myc expression in fibroblasts elevated CDK9 and cyclin T1 protein expression levels since expression was higher in control $myc^{+/+}$ cells than in $myc^{+/+}$ cells with Myc knocked down or in $myc^{-/-}$ cells. Overexpression of MycWT in $myc^{-/-}$ fibroblasts, the Tet21/N neuroblastoma line, and the IMEC line also increases expression of CDK9 and cyclin T1 (Fig. 5D). Again, elevated expression was dependent on the Myc N terminus alone since MycBM and MycΔC but not MycBMΔMBII induced increased CDK9 and cyclin T1 protein expression levels. As with TFIIH, the elevation in CDK9 and cyclin T1 proteins was not mediated by elevated mRNA levels (Fig. 5E).

The Myc-dependent increase in P-TEFb expression correlated with RNA Pol II S2 CTD phosphorylation. S2 phosphorylation was reduced upon the loss of endogenous Myc expression and increased upon the expression of MycWT, MycBM, and MycΔC in rat fibroblasts. S2 phosphorylation was also elevated in neuroblastomas with high N-Myc levels and in IMECs overexpressing MycWT or MycΔC (Fig. 5). Again, we stress that the elevated S2 phosphorylation was found in the total nuclear pool of RNA Pol II.

In summary, both S5 and S2 phosphorylation levels in the RNA Pol II CTD are increased in response to exogenous and endogenous Myc expression. Mutant analysis demonstrated that this is dependent on an intact N terminus but independent of direct Myc DNA binding and target gene expression.

Myc increases the translation rate of cyclin T1 and CDK9. The findings above demonstrate that Myc can increase the expression of a subset of CDKs and cyclins by a posttranscriptional mechanism and independently of Myc target gene expression. Next, we investigated whether these proteins were being upregulated as a result of an increased translation rate or a decreased degradation rate. We used pulse-chase experiments to compare the translation rates and degradation rates of the P-TEFb complex proteins CDK9 and cyclin T1. We measured these rates in the parental $myc^{+/+}$ fibroblasts and $myc^{-/-}$ fibroblasts expressing exogenous MycWT, MycΔMBII, MycBM, MycΔC, and the vector control. The decision to investigate P-TEFb rather than CAK and TFIIH was technical, i.e., in pulse-chase experiments, the simple P-TEFb heterodimer was more amenable to the resolution of individual protein components than the multisubunit TFIIH.

Cells were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine for 0.5, 1, or 1.5 h, followed by a chase with cold amino acids for 2, 4, or 6 h. Cell extracts from each time point were normalized for protein content. To measure total cellular label incorporation for each time point, protein was precipitated using TCA and the counts were quantitated (Fig. 6A). Label incorporation during the pulse was approximately 1.5-fold higher in $myc^{+/+}$ cells than in $myc^{-/-}$ cells ($P > 0.1$). Label incorporation into $myc^{-/-}$ cells expressing MycWT, MycBM, MycΔC, and the vector control was statistically indistinguish-

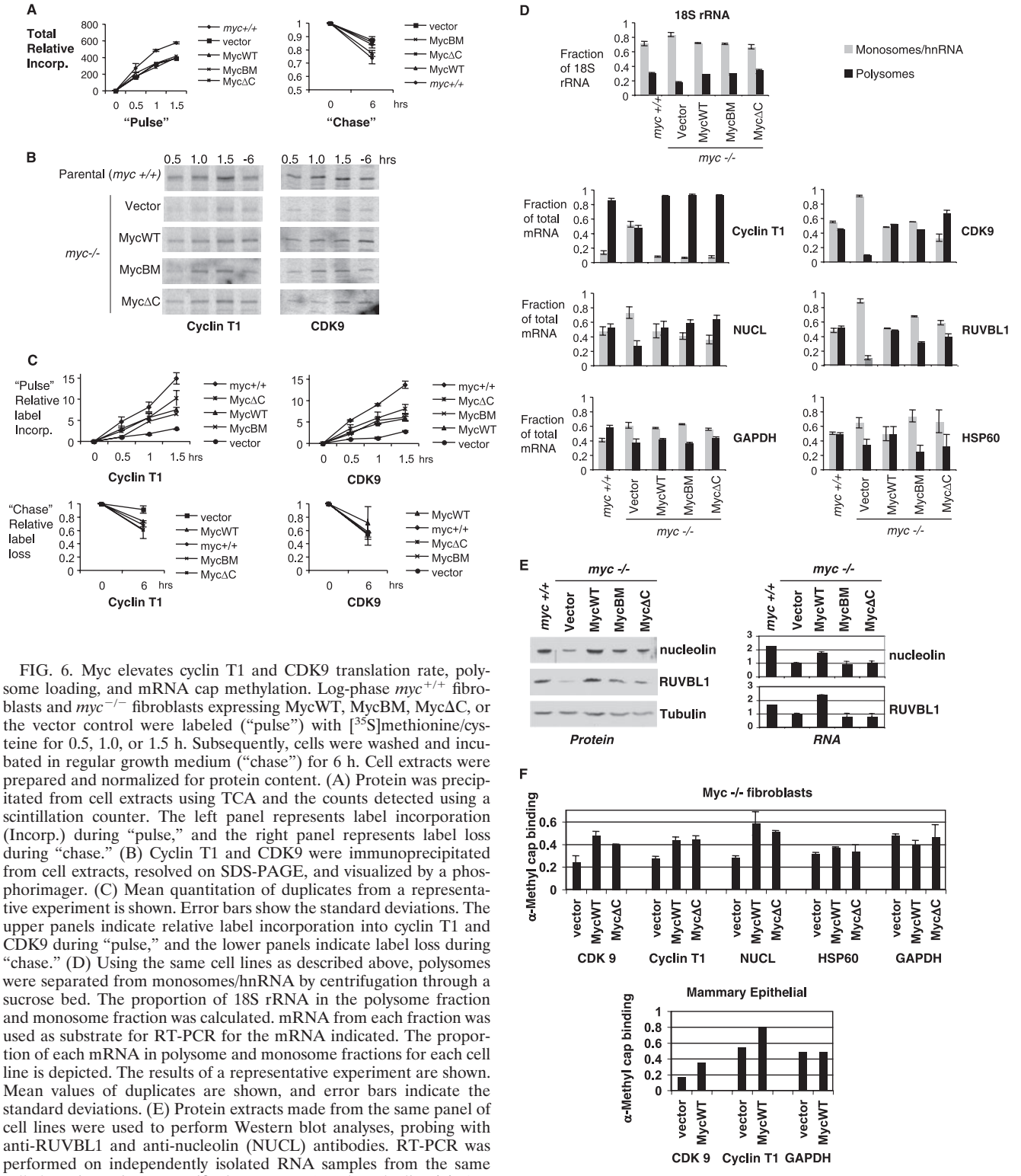

FIG. 6. Myc elevates cyclin T1 and CDK9 translation rate, polysome loading, and mRNA cap methylation. Log-phase $myc^{+/+}$ fibroblasts and $myc^{-/-}$ fibroblasts expressing MycWT, MycBM, MycΔC, or the vector control were labeled ("pulse") with [^{35}S]methionine/cysteine for 0.5, 1.0, or 1.5 h. Subsequently, cells were washed and incubated in regular growth medium ("chase") for 6 h. Cell extracts were prepared and normalized for protein content. (A) Protein was precipitated from cell extracts using TCA and the counts detected using a scintillation counter. The left panel represents label incorporation (Incorp.) during "pulse," and the right panel represents label loss during "chase." (B) Cyclin T1 and CDK9 were immunoprecipitated from cell extracts, resolved on SDS-PAGE, and visualized by a phosphorimager. (C) Mean quantitation of duplicates from a representative experiment is shown. Error bars show the standard deviations. The upper panels indicate relative label incorporation into cyclin T1 and CDK9 during "pulse," and the lower panels indicate label loss during "chase." (D) Using the same cell lines as described above, polysomes were separated from monosomes/hnRNA by centrifugation through a sucrose bed. The proportion of 18S rRNA in the polysome fraction and monosome fraction was calculated. mRNA from each fraction was used as substrate for RT-PCR for the mRNA indicated. The proportion of each mRNA in polysome and monosome fractions for each cell line is depicted. The results of a representative experiment are shown. Mean values of duplicates are shown, and error bars indicate the standard deviations. (E) Protein extracts made from the same panel of cell lines were used to perform Western blot analyses, probing with anti-RUVBL1 and anti-nucleolin (NUCL) antibodies. RT-PCR was performed on independently isolated RNA samples from the same cells by using primers specific for RUVBL1 and nucleolin. (F) Cap methylation was assessed for RNA extracted from $myc^{-/-}$ fibroblasts expressing the vector control, MycWT, and MycΔC and from immortalized mammary epithelial cells expressing the vector control and MycWT. RNA was subjected to immunoprecipitation using anti-2,2,7-methylguanosine antibody. RNA was purified and used as a template for RT-PCR using primers specific for the genes indicated. The quantity of immunoprecipitated RNA is expressed as a fraction of input RNA. The results of representative experiments are shown.

able ($P < 0.1$). Label loss during the chase was lowest for $myc^{-/-}$ cells and highest for $myc^{+/+}$ cells and $myc^{-/-}$ cells expressing MycWT, but the values were statistically indistinguishable ($P > 0.1$). To measure label incorporation into CDK9 and cyclin T1 at each time point, these proteins were

immunoprecipitated and resolved by SDS-PAGE. Label incorporation was detected and quantitated using a phosphorimager (Fig. 6B and C). During the pulse, the rate of label incorporation into cyclin T1 was found to be approximately 4.5-fold higher in $myc^{+/+}$ cells than in $myc^{-/-}$ cells and approximately 2.5-fold higher in $myc^{-/-}$ cells expressing MycWT, MycBM, or MycΔC than in cells expressing the vector control ($P > 0.1$). Similarly, the rate of label incorporation into CDK9 during the pulse was found to be approximately 5.5-fold higher in $myc^{+/+}$ cells than in $myc^{-/-}$ cells and approximately 3-fold higher in $myc^{-/-}$ cells expressing MycWT, MycBM, or MycΔC than in cells expressing the vector control ($P > 0.1$). Label incorporation into cyclin T1 and CDK9 in cells expressing MycΔMBII was indistinguishable from incorporation in vector control cells (see Fig. S1 in the supplemental material). During the chase, label losses from cyclin T1 were similar for all cell lines but actually slowest for $myc^{-/-}$ cells compared to those for all other cell lines tested, i.e., cyclin T1 was most stable in $myc^{-/-}$ cells. Total label loss from CDK9 was statistically indistinguishable for all cell lines ($P < 0.1$). Since cyclin T1 degradation is actually slowest in $myc^{-/-}$ cells and CDK9 degradation rates are equivalent in all cell lines tested, the increased labeled amino acid incorporation during the pulse in cells expressing endogenous Myc, exogenous MycWT, MycBM, and MycΔC can be concluded to be a reflection of an increased translation rate. In addition, the increased translation rate is dependent on MBII, since label incorporation in cyclin T1 and CDK9 is indistinguishable for MycΔMBII and the vector control.

We demonstrated above that Myc upregulates CAK subunits by a posttranscriptional mechanism as with P-TEFb. One or more CAK components may also be upregulated at the level of translation; however, due to the complexity of CAK and TFIIH, it was not possible to investigate the translation rates of these complexes by pulse-chase experiments.

Myc increases polysome loading of cyclin T1, CDK9, and specific Myc target genes. Efficiently translated mRNAs are found to be associated with polysomes rather than monosomes. Since we had shown that CDK9 and cyclin T1 are translated more rapidly in response to Myc, we expected to see a correlative increase in polysome loading. We used centrifugation over a sucrose bed to separate monosomes/hnRNA from polysomes in the fibroblast cell lines (9). Initially, we quantitated 18S rRNA distribution as a measure of ribosome distribution (Fig. 6D). For all cell lines, the 18S rRNA was found predominantly in the monosome fraction. Vector control $myc^{-/-}$ cells had a slight decrease in the proportion of 18S rRNA found in the polysome fraction compared to the Myc-expressing cell lines. mRNA extracted from polysome and monosome fractions was used as a substrate for RT-PCR to look at the distribution of specific messages (Fig. 6D). In $myc^{-/-}$ cells, less than 50% of the cyclin T1 mRNA was associated with polysomes, whereas in $myc^{+/+}$ cells and in $myc^{-/-}$ cells expressing MycWT, MycBR, and MycΔC, approximately 90% of the cyclin T1 mRNA was associated with polysomes. The same trend was found with CDK9. In $myc^{-/-}$ cells, less than 10% of the CDK9 mRNA was associated with polysomes, whereas in all Myc-expressing cell lines, approximately 50% of the CDK9 mRNA was associated with polysomes. The increased association of cyclin T1 and CDK9 with polysomes in response to exogenous and endogenous MycWT and in response to Myc DNA binding mutants is consistent with the increased translation rate of these mRNAs in Myc-expressing cells.

The data above document a novel, target gene-independent activity of Myc; however, we also wanted to explore the possibility that Myc could influence polysome loading of traditional Myc target genes, i.e., increase both the transcription and the translation of some genes. We investigated the polysome loading of three genes which have been established as Myc target genes in rat fibroblasts, the HSP60, RUVBL1, and nucleolin genes (43). We confirmed that the transcription of all three of these genes was upregulated in response to MycWT but not the Myc DNA binding mutants (Fig. 1D and data not shown). For all three mRNAs, polysome loading was increased in $myc^{+/+}$ cells compared to that in $myc^{-/-}$ cells and was increased in response to exogenous MycWT. For HSP60 mRNA, the increase in polysome loading was mild, increasing from 35% in $myc^{-/-}$ cells to 50% in $myc^{+/+}$ cells, and there was no increased polysome loading in response to the DNA binding mutants. However, for nucleolin and RUVBL1, the effect was more significant, and polysome loading was increased in response to the Myc DNA binding mutants as well as the wild-type protein. This differential effect of Myc and Myc mutants is discussed later. Polysome loading for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA was not significantly Myc dependent. In $myc^{-/-}$ cells expressing the vector control and Myc proteins, about 40% of GAPDH mRNA was found to be polysome associated. The parental $myc^{+/+}$ line had a mild increase of up to 60% GAPDH mRNA that was polysome associated.

Since the percentage of polysome loading of nucleolin and RUVBL1 is increased in response to MycWT and Myc DNA binding mutants, the prediction is that the expression of nucleolin and RUVBL1 protein will be increased in response to both MycWT and the Myc DNA binding mutants. We performed Western blot analyses on our panel of rat fibroblast cell lines by using anti-RUVBL1 and antinucleolin antibodies (Fig. 6E). As predicted, we saw increased expression of both proteins in response to endogenous and exogenous MycWT and in response to MycBM and MycΔC compared to that in $myc^{-/-}$ cells. The increase in protein expression was larger in response to MycWT than in response to the Myc DNA binding mutants. This is consistent with the fact that these are also Myc transcriptional target genes, i.e., nucleolin and RUVBL1 mRNAs are elevated in response to MycWT but not the DNA binding mutants (Fig. 6E). Therefore, the increased expression of these proteins in response to MycWT is likely a result of increased transcription and increased polysome loading and translation. The increase in expression is lower in cells expressing MycBM and MycΔC, probably because it is a result of increased translation alone.

Myc increases mRNA cap methylation. Next, we investigated the mechanism by which Myc increases mRNA polysome loading and translation. We found that the nucleocytoplasmic distribution of all the mRNAs discussed above was unaltered by the expression of MycWT or Myc mutants (not shown), and therefore, Myc does not regulate polysome loading and translation of these mRNAs by regulating their nuclear export. We also monitored the expression of a comprehensive panel of translation initiation and elongation factors and found no dif-

ferences between MycBM, MycΔC, and control cells (data not shown).

Translation of mRNA is dependent on the addition of the 7-methylguanosine triphosphate 5′ cap, which occurs most efficiently cotranscriptionally (5, 40). Guanylylation stabilizes mRNA, and methylation permits mRNA translation. (50). Since we had found a group of genes that were equivalently expressed at the mRNA level but that were not equivalently translated, we hypothesized that cap methylation might be regulated.

To measure the proportion of methylated CDK9 and cyclin T1 mRNA, we used an anti-2,2,7-methylguanosine antibody which also binds to 7-methylguanosine (7). RNA isolated from fibroblasts and epithelial cells expressing MycWT, MycΔC, and the vector control was offered as a substrate for immunoprecipitations using the anti-methyl cap antibody. The RNA bound to the anti-methyl cap antibody was isolated, and RT-PCR was used to calculate the antibody-bound fraction (and therefore methylation) of each mRNA relative to that of the input mRNA (Fig. 6F). We found that the proportion of methylated CDK9 and cyclin T1 mRNA was higher in MycWT or MycΔC cells than in the vector control in both fibroblasts and mammary epithelial cells. We also found that nucleolin but not HSP60 mRNA cap methylation was significantly increased in response to MycWT and MycΔC. This is consistent with nucleolin but not HSP60 mRNA polysome loading being well regulated by Myc (Fig. 6D). GAPDH methylation was not regulated by Myc levels for either cell line, which correlates with a lack of stimulation of polysome loading in these cells. Nonspecific binding of mRNA to control antibody beads was uniform and relatively low, <10% of input RNA. We propose that the mechanism by which Myc increases the translation rate of specific messages is by increasing mRNA methyl cap formation for select genes.

The Myc N terminus is recruited to transcription start sites. mRNA capping occurs most efficiently cotranscriptionally, and cap methyltransferase is recruited by the TFIIH-phosphorylated RNA Pol II CTD (25, 48). Therefore, we investigated whether Myc increases TFIIH recruitment to specific genes, in correlation with the increased capping, polysome loading, and translation of these genes. We investigated the in vivo binding of TFIIH to the cyclin T1, cyclin H, and nucleolin transcriptional initiation sites (the CDK9 promoter proved to be refractory to PCR). Using ChIP, we found enhanced recruitment of MAT1 and p62 (subunits of TFIIH) to the cyclin H, cyclin T1, and nucleolin transcription initiation sites in MycWT-, MycBM-and MycΔC-expressing cells compared to that in the vector control (Fig. 7A). In correlation with the fact that MycΔMBII is defective in increasing CDK9 and cyclin T1 translation and expression, enhanced recruitment of TFIIH to transcription initiation sites was not found in cells expressing MycΔMBII (see Fig. S2 in the supplemental material). There was also no Myc-dependent recruitment of TFIIH to the GAPDH transcription initiation sites, which also did not have increased cap methylation and polysome loading.

We had demonstrated that Myc expression increases TFIIH recruitment and mRNA methylation and that this is an activity of the N terminus of Myc independent of the DNA binding domain. We wanted to determine whether Myc could have a direct role in this process by being recruited to the transcription initiation sites via the N terminus. We investigated Myc binding by ChIP using anti-FLAG and anti-Myc antibodies. To confirm that MycΔC was not binding nonspecifically to DNA, we established that MycWT but not MycΔC was recruited to Myc/Max consensus sites in the established HSP60 and nucleolin target genes (Fig. 7B). This confirms that the DNA binding domain is necessary for direct Myc binding to E-box DNA in vivo. In contrast, we found that MycΔMBII, MycBM, and MycΔC were recruited to the transcription initiation sites of cyclin H, cyclin T1, and nucleolin (Fig. 7C and see Fig. S2 in the supplemental material). Therefore, the Myc N terminus can be recruited to these transcriptional initiation sites indirectly and independently of the DNA binding domain. The fact that MycΔMBII is recruited to transcription initiation sites but that MycΔMBII-expressing cells do not have enriched TFIIH recruitment to these sites suggests that Myc-dependent enhanced TFIIH recruitment is dependent on prior MycWT binding. As a negative control, Myc was not found to be recruited to GAPDH initiation sites (Fig. 7C).

Consistent with the model that MBII recruits TFIIH to the transcription initiation sites of certain genes, we found that Myc could be coimmunoprecipitated with CDK7, the kinase subunit of TFIIH (Fig. 7C). Furthermore, MycWT but not MycΔMBII could be coimmunoprecipitated with CDK7 by using an anti-CDK7 antibody, and CDK7 could be coimmunoprecipitated with MycWT but not MycΔMBII by using an anti-Myc antibody. Endogenous CDK7 could also be immunoprecipitated with endogenous Myc protein from 293 cells (Fig. 7D).

DISCUSSION

Myc increases CTD kinase activity. Here, we report that Myc induces the expression of both CDK7 and CDK9 kinases, which phosphorylate the RNA Pol II CTD as components of TFIIH and P-TEFb, respectively, and that there is a concomitant increase in the phosphorylation of their respective substrates, S5 and S2, in the CTD. All of the activities we demonstrate for MycWT are manifested by the Myc transactivation domain alone and thus are within the repertoire of normal Myc function but independent of target gene activation. We show that these kinase activities are induced in response to c-Myc and N-Myc in fibroblasts, epithelial cells, and neuroblastoma cells. We also demonstrate that Myc can promote TFIIH recruitment, cap methylation, and translation of a subset of genes. Myc can be recruited to transcription initiation sites via the N terminus alone. Myc can also bind CDK7 and promote TFIIH recruitment in an MBII-dependent manner and therefore may have a direct role in this process. The fact that Myc can significantly increase the level of CTD phosphorylation in the total pool of RNA Pol II, rather than at specific promoters, is evidence of the potent effect of the Myc N terminus. RNA Pol II phosphorylation governs many aspects of transcription and cotranscriptional processing (5, 29, 52), and the finding that Myc can substantially increase CTD phosphorylation is suggestive of a global role for Myc in these processes.

It is interesting that Myc can directly enhance the recruitment of TFIIH to both the cyclin H and cyclin T1 promoters (Fig. 7). Myc induces posttranscriptional elevation in these cyclin protein levels as well as other CDK components. The increased levels of these CDK/cyclin complexes could syner-

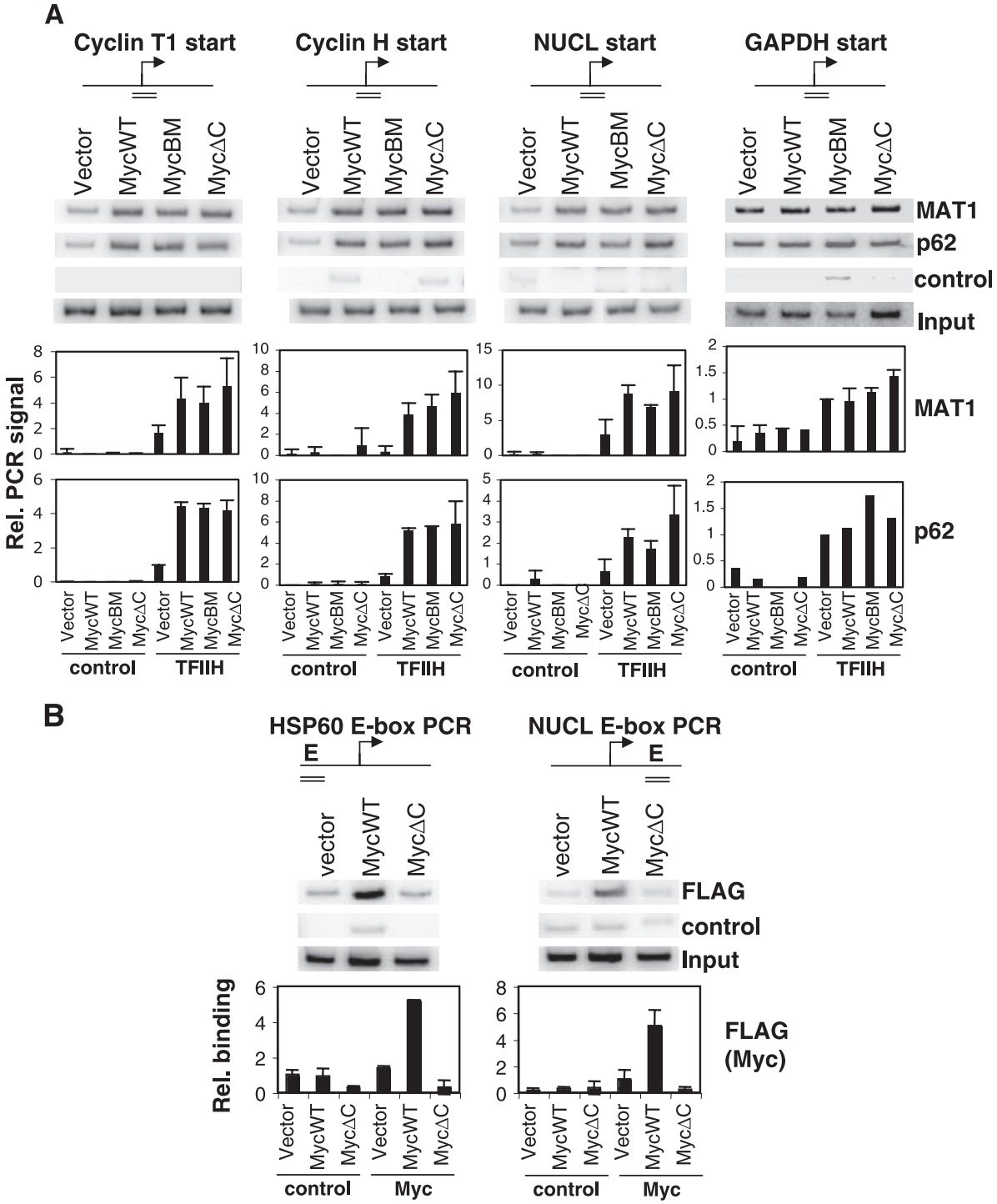

FIG. 7. Myc promotes TFIIH binding and binds to the transcription initiation sites via the N terminus. ChIP was performed on *myc*$^{-/-}$ fibroblasts expressing MycWT, MycBM, MycΔC, or the vector control. (A) Complexes were immunoprecipitated with antibodies raised against the TFIIH subunits MAT1 and p62 or control antibody. Coprecipitated DNA was used as a template for PCR using primers specific for cyclin T1, cyclin H, nucleolin (NUCL), and GAPDH transcriptional start sites. PCR products were labeled, run on a gel, and visualized by a phosphorimager (upper panels). PCR products were quantitated and normalized to input PCR, and the relative (Rel.) signal is depicted. The mean values for at least two independent experiments are shown in the graphs. Error bars show the standard deviations. (B) Complexes were immunoprecipitated with anti-FLAG (Myc) or control antibody. PCR was carried out using primers specific for HSP60 and nucleolin E boxes. (C) Complexes were immunoprecipitated with antibodies raised against the FLAG (Myc) tag and Myc or control antibody. PCR was carried out using primers specific for cyclin T1, cyclin H, nucleolin, and GAPDH transcription start sites. (D) 293 cells were transfected with MycWT, MycΔMBII, and CDK7 as indicated. Immunoprecipitation was carried out with cell extracts using anti-CDK7 (left panel) or anti-Myc (middle panel). Immunoprecipitated proteins and cell extracts (right panel) were subjected to Western blotting using anti-CDK7 and anti-Myc antibodies. (E) Immunoprecipitation was carried out using anti-c-Myc antibodies or control antibodies on untransfected 293 cells. Immunoprecipitated protein and cell extracts were subjected to Western blotting using anti-CDK7 and anti-Myc antibodies.

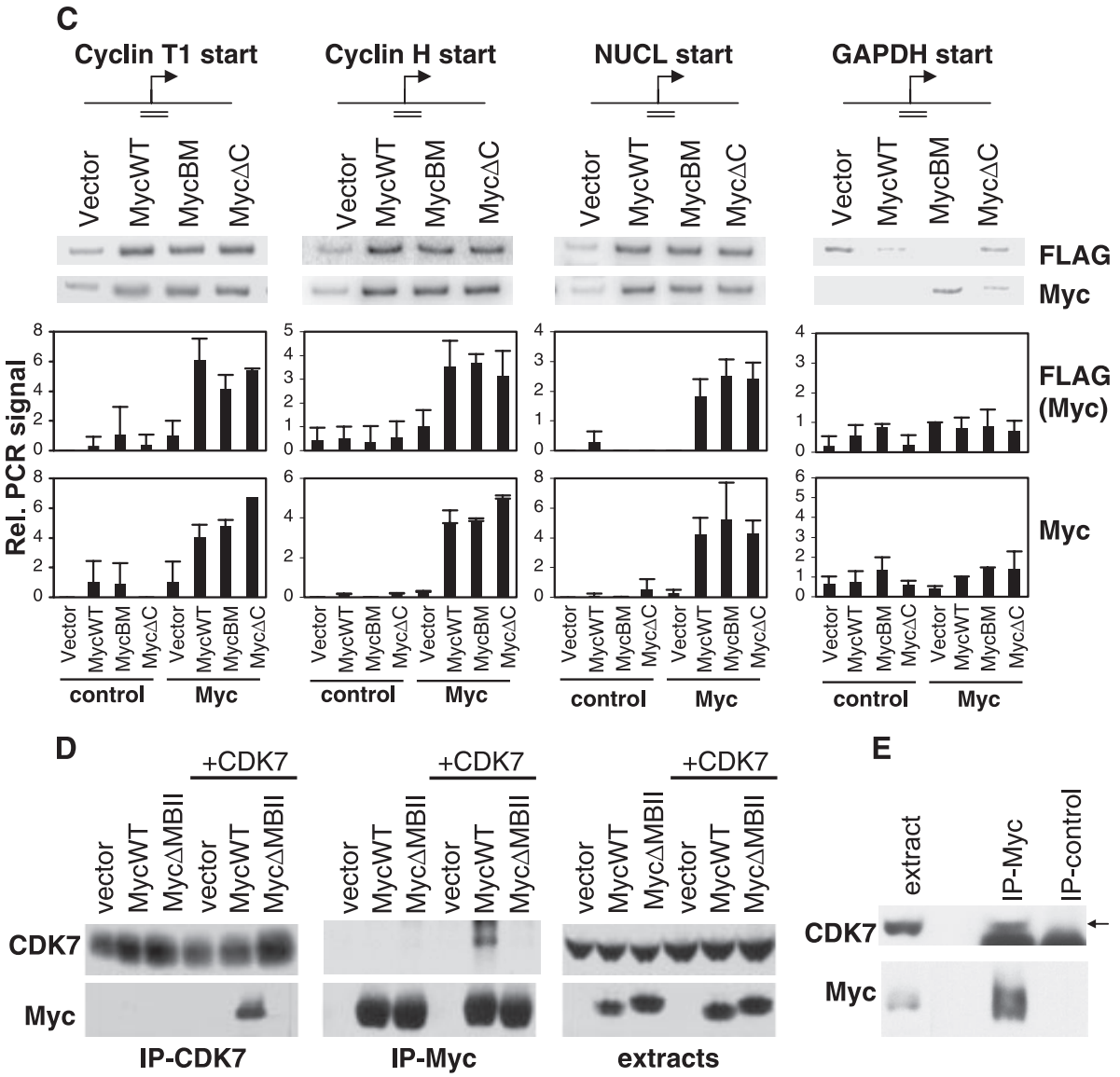

FIG. 7—*Continued.*

gize with Myc-induced recruitment in a positive feedback loop to further enhance cellular CTD kinase levels.

Myc activates CAK and promotes proliferation independently of target gene expression. Myc-driven cell proliferation is essential for its role in development, normal cell physiology, and cell transformation (8, 41). One of the most surprising findings of this study is that the N terminus of Myc has sufficient biological activity to actually rescue a large fraction of the proliferation defect of *myc* null fibroblasts. Although MycΔC and MycBM do not regulate the transcription of Myc target genes, they have sufficient activity to rescue the cell doubling time from 40 h to 26 h. MycΔC and MycBM were used as complementary mutants since MycΔC cannot bind to Max, whereas MycBM can. Since both mutants have an increased proliferation rate, we can rule out disturbance of the Max network as being responsible for this Myc N-terminal activity. In addition, the N-terminal MycBMΔMBII mutant cannot induce cell proliferation despite being able to bind to Max equivalently to MycBM, thus localizing the proliferative activity to a critical N-terminal domain.

Cell proliferation is driven by a cycle of CDK activation and degradation. CAK phosphorylates other CDKs, increasing the activity and stability of cell cycle CDK/cyclin complexes (23). Previously, MycWT has been shown to increase cellular CAK activity (32). In this paper, we show that MycWT and DNA binding mutants increase the expression and activity of CAK independently of target gene expression. In addition, we show that CDK7 is rate limiting for CDK phosphorylation and cell proliferation. Since CAK is also a component of TFIIH, it is not possible to deduce whether inhibition of CDK7 reduces cell proliferation because it inhibits TFIIH-induced RNA Pol II phosphorylation or CAK-induced CDK phosphorylation. Indeed, Myc may induce proliferation via CDK7 by activating both TFIIH and CAK.

A previous report which addressed the mechanism by which Myc drives proliferation demonstrated that DNA binding and dimerization with Max are necessary for Myc to maintain cells in S phase following serum withdrawal (2), and indeed, our non-DNA binding mutants behave similarly under serum-starved conditions (unpublished data). We do not believe that this previous study conflicts with our own because our studies used log-phase cells, i.e., subconfluent cultures grown in serum. These two studies reveal redundant mechanisms by which Myc can drive proliferation, both dependent and independent of gene regulation.

Myc regulation of mRNA cap methylation. In this paper, we report the first example of the regulation of mammalian mRNA cap methylation. Yeast mutant analysis and biochemical assays have demonstrated that mRNA guanylylation is necessary for stabilizing mRNA, and methylation of the guanylyl group is necessary to permit translation of the mRNA (40, 50, 51). Since these two steps are carried out by separate enzymes and the addition of guanylyl and methyl groups occurs with a time delay in vitro, it has been proposed that differential regulation of these enzymes may exist (38, 51). Here, we show that Myc can increase the methylation of specific messages and that this is a property of the Myc N terminus alone. Myc is recruited to transcription initiation sites accompanied by TFIIH in an MBII-dependent manner. The TFIIH-phosphorylated CTD has been reported to recruit methyltransferase, but it remains possible that Myc may stimulate the methyltransferase either via TFIIH or more directly. It is interesting to draw comparisons between our study of Myc and a recent study of GAL4 (39). A GAL4 mutant was demonstrated to increase CTD phosphorylation and translation rather than transcription of target genes, and we propose that methylation of these target gene mRNAs may be responsible for the increased GAL4 target translation rate.

TFIIH recruitment is also associated with increased transcription and mRNA guanylylation, both of which result in increased mRNA abundance. However, in our system, although we see Myc-dependent recruitment of TFIIH to some promoters, we do not see an increase in mRNA abundance for these genes. There are many explanations that could account for this observation, the simplest being that transcription, capping, and cap methylation may be promoter dependent and differentially sensitive to the TFIIH-phosphorylated CTD.

MycWT upregulates the transcription and translation of a subset of Myc target genes. We found that two traditional Myc target genes, the nucleolin and RUVBL1 genes, had increased cap methylation and polysome loading in response to MycWT and the DNA binding mutants. Consistent with increased polysome loading, we found that nucleolin and RUVBL1 protein expression levels are also elevated in response to the expression of MycWT, MycBM, and MycΔC. The increase in protein expression is larger in response to MycWT than to the DNA binding mutants, consistent with these genes having both increased transcription and increased translation in response to MycWT. Thus, Myc may increase the protein concentration of a large subset of genes by upregulating both their transcription and their translation. Another Myc target gene, the HSP60 gene, did not exhibit a robust increase in cap methylation and polysome loading in response to the Myc DNA binding mutants, revealing that the recruitment of Myc to an E box is not sufficient to stimulate cap methylation. A small increase in HSP60 polysome loading was observed in response to MycWT, and this may be a reflection of the increased expression of eukaryotic initiation factors 4E and 4G induced by MycWT (but not Myc DNA binding mutants) (47). Indeed, MycWT expression has been reported previously to increase the polysome loading of specific mRNAs (36). More global studies will be required to determine which target genes are cap methylated in response to Myc.

Summary. Myc is a potent and pleiotropic effector of cellular growth and a well-established transcription factor. Here, we report that Myc activity extends beyond transcriptional regulation of target genes to a general role in promoting global phosphorylation of the RNA Pol II CTD. This activity is likely to have broad-ranging effects on transcription and mRNA metabolism, such as increased cap methylation on some mRNAs. The combination of this global activity with the transactivation of numerous target genes may account for the prominent role of Myc as an oncogene.

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REFERENCES

1. **Adhikary, S., F. Marinoni, A. Hock, E. Hulleman, N. Popov, R. Beier, S. Bernard, M. Quarto, M. Capra, S. Goettig, U. Kogel, M. Scheffner, K. Helin, and M. Eilers.** 2005. The ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation. Cell **123:**409–421.
2. **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. **12:**5083–5087.
3. **Arabi, A., S. Wu, K. Ridderstrale, H. Bierhoff, C. Shiue, K. Fatyol, S. Fahlen, P. Hydbring, O. Soderberg, I. Grummt, L. G. Larsson, and A. P. Wright.** 2005. c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. Nat. Cell Biol. **7:**303–310.
4. **Armelin, H. A., M. C. Armelin, K. Kelly, T. Stewart, P. Leder, B. H. Cochran, and C. D. Stiles.** 1984. Functional role for c-myc in mitogenic response to platelet-derived growth factor. Nature **10:**55–660.
5. **Bentley, D. L.** 2005. Rules of engagement: co-transcriptional recruitment of pre-mRNA processing factors. Curr. Opin. Cell Biol. **17:**251–256.
6. **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.
7. **Bochnig, P., R. Reuter, P. Bringmann, and R. Luhrmann.** 1987. A monoclonal antibody against 2,2,7-trimethylguanosine that reacts with intact, class U, small nuclear ribonucleoproteins as well as with 7-methylguanosine-capped RNAs. Eur. J. Biochem. **68:**61–467.
8. **Boxer, L. M., and C. V. Dang.** 2001. Translocations involving c-myc and c-myc function. Oncogene **20:**5595–5610.
9. **Brewer, G., and J. Ross.** 1990. Messenger RNA turnover in cell-free extracts. Methods Enzymol. **181:**202–209.
10. **Cawley, S., S. Bekiranov, H. H. Ng, P. Kapranov, E. A. Sekinger, D. Kampa, A. Piccolboni, V. Sementchenko, J. Cheng, A. J. Williams, R. Wheeler, B. Wong, J. Drenkow, M. Yamanaka, S. Patel, S. Brubaker, H. Tammana, G. Helt, K. Struhl, and T. R. Gingeras.** 2004. Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. Cell **116:**499–509.
11. **Cho, E. J., T. Takagi, C. R. Moore, and S. Buratowski.** 1997. mRNA capping enzyme is recruited to the transcription complex by phosphorylation of the RNA polymerase II carboxy-terminal domain. Genes Dev.**1:**319–3326.
12. **Cole, M. D.** 1986. The myc oncogene: its role in transformation and differentiation. Annu. Rev. Genet. **0:**61–385.
13. **Coppola, J. A., A. S. Field, and D. S. Luse.** 1983. Promoter-proximal pausing by RNA polymerase II in vitro: transcripts shorter than 20 nucleotides are not capped. Proc. Natl. Acad. Sci. USA **80:**1251–1255.
14. **Cowling, V. H., S. Chandriani, M. L. Whitfield, and M. D. Cole.** 2006. A conserved Myc protein domain, MBIV, regulates DNA binding, apoptosis, transformation, and G_2 arrest. Mol. Cell. Biol. **26:**4226–4239.
15. **Cowling, V. H., and M. D. Cole.** 2006. Mechanism of transcriptional activation by the Myc oncoproteins. Semin. Cancer Biol. **16:**242–252.

16. **Dang, C. V., K. A. O'Donnell, K. I. Zeller, T. Nguyen, R. C. Osthus, and F. Li.** 2006. The c-Myc target gene network. Semin. Cancer Biol. **16:**253–264.
17. **DiRenzo, J., S. Signoretti, N. Nakamura, R. Rivera-Gonzalez, W. Sellers, M. Loda, and M. Brown.** 2002. Growth factor requirements and basal phenotype of an immortalized mammary epithelial cell line. Cancer Res. **62:**89–98.
18. **Eberhardy, S. R., and P. J. Farnham.** 2002. Myc recruits P-TEFb to mediate the final step in the transcriptional activation of the cad promoter. J. Biol. Chem. **277:**40156–40162.
19. **Eilers, M., D. Picard, K. R. Yamamoto, and J. M. Bishop.** 1989. Chimaeras of Myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. Nature **340:**66–68.
20. **Fernandez, P. C., S. R. Frank, L. Wang, M. Schroeder, S. Liu, J. Greene, A. Cocito, and B. Amati.** 2003. Genomic targets of the human c-Myc protein. Genes Dev. **17:**1115–1129.
21. **Grandori, C., N. Gomez-Roman, Z. A. Felton-Edkins, C. Ngouenet, D. A. Galloway, R. N. Eisenman, and R. J. White.** 2005. c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. Nat. Cell Biol. **7:**311–318.
22. **Grewal, S. S., L. Li, A. Orian, R. N. Eisenman, and B. A. Edgar.** 2005. Myc-dependent regulation of ribosomal RNA synthesis during Drosophila development. Nat. Cell Biol. **7:**295–302.
23. **Harper, J. W., and S. J. Elledge.** 1998. The role of Cdk7 in CAK function, a retro-retrospective. Genes Dev. **12:**285–289.
24. **Kim, S. Y., A. Herbst, K. A. Tworkowski, S. E. Salghetti, and W. P. Tansey.** 2003. Skp2 regulates Myc protein stability and activity. Mol. Cell **11:**1177–1188.
25. **Komarnitsky, P., E. J. Cho, and S. Buratowski.** 2000. Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. Genes Dev. **14:**2452–2460.
26. **Li, Z., S. Van Calcar, C. Qu, W. K. Cavenee, M. Q. Zhang, and B. Ren.** 2003. A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. Proc. Natl. Acad. Sci. USA **100:**8164–8169.
27. **Lutz, W., M. Stohr, J. Schurmann, A. Wenzel, A. Lohr, and M. Schwab.** 1996. Conditional expression of N-myc in human neuroblastoma cells increases expression of alpha-prothymosin and ornithine decarboxylase and accelerates progression into S-phase early after mitogenic stimulation of quiescent cells. Oncogene **13:**803–812.
28. **Mäkelä, T. P., J. D. Parvin, J. Kim, L. J. Huber, P. A. Sharp, and R. A. Weinberg.** 1995. A kinase-deficient transcription factor TFIIH is functional in basal and activated transcription. Proc. Natl. Acad. Sci. USA **92:**5174–5178.
29. **Maniatis, T., and R. Reed.** 2002. An extensive network of coupling among gene expression machines. Nature **416:**499–506.
30. **Mao, D. Y., J. D. Watson, P. S. Yan, D. Barsyte-Lovejoy, F. Khosravi, W. W. Wong, P. J. Farnham, T. H. Huang, and L. Z. Penn.** 2003. Analysis of Myc bound loci identified by CpG island arrays shows that Max is essential for Myc-dependent repression. Curr. Biol. **13:**882–886.
31. **Mateyak, M. K., A. J. Obaya, S. Adachi, and J. M. Sedivy.** 1997. Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. Cell Growth Differ. **8:**1039–1048.
32. **Mateyak, M. K., A. J. Obaya, and J. M. Sedivy.** 1999. c-Myc regulates cyclin D-Cdk4 and -Cdk6 activity but affects cell cycle progression at multiple independent points. Mol. Cell. Biol. **19:**4672–4683.
33. **McCracken, S., N. Fong, E. Rosonina, K. Yankulov, G. Brothers, D. Siderovski, A. Hessel, S. Foster, S. Shuman, and D. L. Bentley.** 1997. 5′-capping enzymes are targeted to pre-mRNA by binding to the phosphorylated carboxy-terminal domain of RNA polymerase II. Genes Dev. **11:**3306–3318.
34. **McMahon, S. B., H. A. Van Buskirk, K. A. Dugan, T. D. Copeland, and M. D. Cole.** 1998. The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. Cell **94:**363–374.
35. **McMahon, S. B., M. A. Wood, and M. D. Cole.** 2000. The essential cofactor TRRAP recruits the histone acetyltransferase hGCN5 to c-Myc. Mol. Cell. Biol. **20:**556–562.
36. **Mezquita, P., S. S. Parghi, K. A. Brandvold, and A. Ruddell.** 2005. Myc regulates VEGF production in B cells by stimulating initiation of VEGF mRNA translation. Oncogene **24:**889–901.
37. **Morgan, D. O.** 1995. Principles of CDK regulation. Nature **374:**131–134.
38. **Moteki, S., and D. Price.** 2002. Functional coupling of capping and transcription of mRNA. Mol. Cell **10:**599–609.
39. **Muratani, M., C. Kung, K. M. Shokat, and W. P. Tansey.** 2005. The F box protein Dsg1/Mdm30 is a transcriptional coactivator that stimulates Gal4 turnover and cotranscriptional mRNA processing. Cell **120:**887–899.
40. **Muthukrishnan, S., G. W. Both, Y. Furuichi, and A. J. Shatkin.** 1975. 5′-terminal 7-methylguanosine in eukaryotic mRNA is required for translation. Nature **255:**33–37.
41. **Obaya, A. J., M. K. Mateyak, and J. M. Sedivy.** 1999. Mysterious liaisons: the relationship between c-Myc and the cell cycle. Oncogene **18:**2934–2941.
42. **O'Brien, T., S. Hardin, A. Greenleaf, and J. T. Lis.** 1994. Phosphorylation of RNA polymerase II C-terminal domain and transcriptional elongation. Nature **370:**75–77.
43. **O'Connell, B. C., A. F. Cheung, C. P. Simkevich, W. Tam, X. Ren, M. K. Mateyak, and J. M. Sedivy.** 2003. A large scale genetic analysis of c-Myc-regulated gene expression patterns. J. Biol. Chem. **278:**12563–12573.
44. **Orian, A., B. van Steensel, J. Delrow, H. J. Bussemaker, L. Li, T. Sawado, E. Williams, L. W. Loo, S. M. Cowley, C. Yost, S. Pierce, B. A. Edgar, S. M. Parkhurst, and R. N. Eisenman.** 2003. Genomic binding by the Drosophila Myc, Max, Mad/Mnt transcription factor network. Genes Dev. **17:**1101–1114.
45. **Pelengaris, S., M. Khan, and G. Evan.** 2002. c-MYC: more than just a matter of life and death. Nat. Rev. Cancer **2:**764–776.
46. **Rasmussen, E. B., and J. T. Lis.** 1993. In vivo transcriptional pausing and cap formation on three Drosophila heat shock genes. Proc.Natl.Acad.Sci. USA **0:**923–7927.
47. **Schmidt, E. V.** 2004. The role of c-myc in regulation of translation initiation. Oncogene **23:**3217–3221.
48. **Schroeder, S. C., B. Schwer, S. Shuman, and D. Bentley.** 2000. Dynamic association of capping enzymes with transcribing RNA polymerase II. Genes Dev. **14:**2435–2440.
49. **Schuhmacher, M., F. Kohlhuber, M. Holzel, C. Kaiser, H. Burtscher, M. Jarsch, G. W. Bornkamm, G. Laux, A. Polack, U. H. Weidle, and D. Eick.** 2001. The transcriptional program of a human B cell line in response to Myc. Nucleic Acids Res. **29:**397–406.
50. **Schwer, B., X. Mao, and S. Shuman.** 1998. Accelerated mRNA decay in conditional mutants of yeast mRNA capping enzyme. Nucleic Acids Res. **26:**2050–2057.
51. **Schwer, B., N. Saha, X. Mao, H. W. Chen, and S. Shuman.** 2000. Structure-function analysis of yeast mRNA cap methyltransferase and high-copy suppression of conditional mutants by AdoMet synthase and the ubiquitin conjugating enzyme Cdc34p. Genetics **155:**1561–1576.
52. **Sims, R. J., III, S. S. Mandal, and D. Reinberg.** 2004. Recent highlights of RNA-polymerase-II-mediated transcription. Curr.Opin.Cell Biol.**6:**63–271.
53. **Sommer, A., K. Bousset, E. Kremmer, M. Austen, and B. Luscher.** 1998. Identification and characterization of specific DNA-binding complexes containing members of the Myc/Max/Mad network of transcriptional regulators. J. Biol. Chem. **273:**6632–6642.
54. **Stone, J., T. de Lange, G. Ramsay, E. Jakobovits, J. M. Bishop, H. Varmus, and W. Lee.** 1987. Definition of regions in human c-*myc* that are involved in transformation and nuclear localization. Mol. Cell. Biol. **7:**1697–1709.
55. **Takagi, T., C. R. Moore, F. Diehn, and S. Buratowski.** 1997. An RNA 5′-triphosphatase related to the protein tyrosine phosphatases. Cell **89:**867–873.
56. **von der Lehr, N., S. Johansson, S. Wu, F. Bahram, A. Castell, C. Cetinkaya, P. Hydbring, I. Weidung, K. Nakayama, K. I. Nakayama, O. Soderberg, T. K. Kerppola, and L. G. Larsson.** 2003. The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription. Mol.Cell **1:**189–1200.
57. **Welcker, M., A. Orian, J. Jin, J. E. Grim, J. W. Harper, R. N. Eisenman, and B. E. Clurman.** 2004. The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. Proc. Natl. Acad. Sci. USA **101:**9085–9090.
58. **Yada, M., S. Hatakeyama, T. Kamura, M. Nishiyama, R. Tsunematsu, H. Imaki, N. Ishida, F. Okumura, K. Nakayama, and K. I. Nakayama.** 2004. Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. EMBO J. **23:**2116–2125.
59. **Yue, Z., E. Maldonado, R. Pillutla, H. Cho, D. Reinberg, and A. J. Shatkin.** 1997. Mammalian capping enzyme complements mutant Saccharomyces cerevisiae lacking mRNA guanylyltransferase and selectively binds the elongating form of RNA polymerase II. Proc. Natl. Acad. Sci. USA **94:**12898–12903.