

c-Myc Mediates Activation of the *cad* Promoter via a Post-RNA Polymerase II Recruitment Mechanism*

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Scott R. Eberhardy and Peggy J. Farnham‡

From the University of Wisconsin Medical School, Madison, Wisconsin 53706

The c-Myc protein is a site-specific DNA-binding transcription factor that is up-regulated in a number of different cancers. We have previously shown that binding of Myc correlates with increased transcription of the *cad* promoter. We have now further investigated the mechanism by which Myc mediates transcriptional activation of the *cad* gene. Using a chromatin immunoprecipitation assay, we found high levels of RNA polymerase II bound to the *cad* promoter in quiescent NIH 3T3 cells and in differentiated U937 cells, even though the promoter is inactive. However, chromatin immunoprecipitation with an antibody that recognizes the hyperphosphorylated form of the RNA polymerase II carboxyl-terminal domain (CTD) revealed that phosphorylation of the CTD does correlate with c-Myc binding and *cad* transcription. We have also found that the c-Myc transactivation domain interacts with cdk9 and cyclin T1, components of the CTD kinase P-TEFb. Furthermore, activator bypass experiments have shown that direct recruitment of cyclin T1 to the *cad* promoter can substitute for c-Myc to activate the promoter. In summary, our results suggest that c-Myc activates transcription of *cad* by stimulating promoter clearance and elongation, perhaps via recruitment of P-TEFb.

c-Myc is a site-specific DNA-binding transcription factor that is conserved throughout evolution. Deregulated expression of c-Myc occurs in many cancers, including lymphomas, breast, colon, and liver cancer. c-Myc is overexpressed in neoplasia by a number of different mechanisms, including gene amplification, translocation, retroviral insertion, and activation of pathways upstream of c-Myc expression (for review, see Ref. 1). A number of studies have established the importance of c-Myc in neoplastic transformation. For example, it has long been known that co-transfection of c-Myc and Ras in rat embryo fibroblasts causes transformation in these cells (2). Additionally, targeted overexpression of c-Myc to various tissues of transgenic mice can cause increased tumor incidence (3–6).

c-Myc is a member of the helix-loop-helix leucine zipper family of transcription factors. Helix-loop-helix leucine zipper proteins bind to a DNA motif called an E box, which consists of the core consensus sequence CACGTG (7). Efficient binding of c-Myc to an E box can only occur when c-Myc forms het-

erodimers with the protein Max, another helix-loop-helix leucine zipper protein (8). When Myc/Max heterodimers bind to DNA, c-Myc can activate transcription through a transcriptional activation domain (TAD)¹ in the amino-terminal region of the protein (9). c-Myc function is antagonized by the Mad proteins, which can also dimerize with Max and bind to E boxes (10). Mad proteins repress transcription through their ability to recruit the mSin3 corepressor complex (11). Although c-Myc is highly expressed in proliferating cells, Mad proteins are found mainly in quiescent and differentiated cells. Thus, the relative abundance of c-Myc and Mad proteins in a cell is thought to dictate gene expression.

Recent advances in microarray technology has allowed genome-wide studies of mRNA transcripts responsive to transcription factors, and a number of such experiments have been done to examine which genes are responsive to c-Myc (12–14). These studies have confirmed the Myc responsiveness of a number of proposed target genes, such as *ornithine decarboxylase (ode)* (15), *nucleolin* (16), *cyclin D2 (ccnd2)* (17), and *cdk4* (18). Additionally, genes involved in general responses such as glycolysis and protein synthesis appear to be influenced by c-Myc (19, 20). However, mRNA studies do not conclusively establish the identity of a c-Myc target promoter. It is likely that many mRNAs that respond to c-Myc are indirectly affected by Myc-mediated changes in signaling pathways. To establish a promoter as a c-Myc target, it is necessary to show that binding of c-Myc causes changes in promoter activity. However, a difficulty in studying c-Myc target genes is that overexpression of c-Myc in cells results in only modest increases in promoter activity, typically around 2–5-fold. This may be due to several factors, including the presence of highly abundant proteins, such as USF, which also bind to target gene E boxes and may interfere with c-Myc binding, or a requirement for a coactivator which is at limiting concentrations in the cell.

We have previously shown that the *cad* promoter meets all the criteria of a true c-Myc target. The *cad* gene encodes the trifunctional enzyme carbamoyl-phosphate synthase/aspartate transcarbamoylase/dihydroorotase, which is required for the first three rate-limiting steps of pyrimidine biosynthesis. Expression of *cad* is high in proliferating cells and low in quiescent and differentiated cells, which closely correlates with c-Myc expression. The *cad* promoter contains a conserved E box downstream of the transcription start site which we have shown to be essential for growth regulation (21). Importantly, we have used chromatin immunoprecipitation (ChIP) studies to

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‡ To whom correspondence should be addressed: McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, WI 53706. Tel.: 608-262-2071; Fax: 608-262-2824; E-mail: farnham@oncology.wisc.edu.

¹ The abbreviations used are: TAD, transactivation domain; *cad*, carbamoyl-phosphate synthase/aspartate transcarbamoylase/dihydroorotase; ChIP, chromatin immunoprecipitation; RNAP II, RNA polymerase II; CTD, carboxyl-terminal domain; P-TEFb, positive elongation factor b; HAT, histone acetyltransferase; tert, telomerase reverse transcriptase; cdk, cyclin-dependent kinase; CMV, cytomegalovirus; GST, glutathione S-transferase; HIV, human immunodeficiency virus; RT, reverse transcriptase; IP, immunoprecipitation.

show that c-Myc binds to the *cad* promoter during peak levels of transcription and is not bound when *cad* is not expressed (22). Finally, *cad* is one of the few proposed c-Myc target genes whose expression is decreased in c-Myc null cells (23).

Transcription of protein-coding genes by RNA polymerase II (RNAP II) is believed to involve three main events (24). First, chromatin modification and remodeling of the promoter occurs to facilitate transcription factor binding. Then, general transcription factors bind to the promoter to recruit RNAP II to the promoter to form the preinitiation complex. Finally, RNAP II clears the promoter and begins elongation of mRNA. Stimulation of elongation appears to require phosphorylation of the RNAP II large subunit carboxyl-terminal domain (CTD), which contains multiple tandem repeats of the amino acid sequence YSPTSPS. A number of kinases have the ability to phosphorylate the RNAP II CTD, including the cyclin-cdk complexes cyclin H-cdk7, cyclin C-cdk8, and cyclin T-cdk9. Cyclin H and cdk7 are part of the general transcription factor TFIIF, which has been shown to be important in stimulating elongation (25). Cyclin C and cdk8 are part of the mediator complex, and while their role in transcriptional elongation has not been fully elucidated, they have been shown to co-purify with RNAP II (26). Cdk9 and the cyclin T proteins, which consist of cyclin T1 and cyclins T2a and T2b, are part of a complex known as P-TEFb (for positive transcription elongation factor b), which has been shown to be important for HIV transcriptional elongation through its association with Tat (27).

Current models for c-Myc function suggest that transcriptional control of c-Myc target genes may occur through chromatin modification. c-Myc has recently been shown to interact with the protein TRRAP, which associates with the SAGA complex that contains Gcn5, a protein that has histone acetyltransferase (HAT) activity (28). In contrast, Mad proteins interact with histone deacetylases through association with the mSin3 corepressor complex (29). These observations have led to a model in which c-Myc target genes are repressed by Mad due to deacetylation of histones. Replacement of Mad/Max by Myc/Max is then proposed to result in recruitment of HAT activity and acetylation of histones, subsequently allowing RNAP II to bind and initiate transcription. In support of this model, recent studies have shown that at some c-Myc target promoters, such as nucleolin, changes in histone acetylation do correlate with binding of c-Myc (30). Also, a detailed analysis of the *cyclin D2* promoter using chromatin immunoprecipitation indicates that c-Myc and TRRAP bind to the *cyclin D2* promoter when the gene is transcribed whereas Mad and histone deacetylases 1 bind to the promoter when the gene is turned off (31). Finally, a study of the telomerase reverse transcriptase (*tert*) promoter has also shown that histone acetylation correlates with transcription and c-Myc binding (32).

Although Myc clearly regulates the expression of some, and perhaps most, target promoters by recruiting TRRAP and causing an increase in acetylated histones, we have not seen large changes in the levels of histone acetylation at the *cad* promoter (33). In fact, expression studies using c-Myc constructs containing or lacking the TRRAP-binding domain indicate that c-Myc-mediated activation of the *cad* promoter does not require recruitment of TRRAP and the associated histone acetyltransferases.² These findings, in combination with our previous studies showing that the *cad* promoter contains high levels of acetylated histones at all times, have led us to investigate other possible mechanisms through which c-Myc may activate transcription. Using the ChIP assay, we have now examined RNAP

II binding at the *cad* gene. We have found that RNAP II is bound to the *cad* promoter at all times but levels of the hyperphosphorylated form of RNAP II change in correlation with c-Myc binding. We also demonstrate an interaction between the c-Myc TAD and P-TEFb, and activator bypass experiments show that cyclin T1 can substitute for c-Myc to activate the *cad* promoter. These results suggest that c-Myc functions to stimulate *cad* transcription by recruitment of the P-TEFb complex.

MATERIALS AND METHODS

Cell Culture and Transfections—NIH 3T3 cell cultures were maintained and synchronized as previously described (35). Briefly, 6–8 × 10⁶ cells were seeded into 500-cm² tissue culture dishes (1–2 dishes were used per antibody per time point in the formaldehyde cross-linking experiments) and incubated in starvation medium for 48–72 h. Cells were then either stimulated to enter the cell cycle by the addition of stimulation medium for 4 h (early G₁ phase) or 12 h (G₁/S phase) prior to cross-linking. Progression of the cells through the cell cycle was measured by flow cytometric analysis of propidium iodide-stained cells as described previously (36). Data were acquired on a FACScan flow cytometer (Becton Dickinson) using CellQuest acquisition and analysis software (data not shown). For *cad*-luciferase co-transfections, 1 × 10⁵ cells were co-transfected with *cad* 3G4luc and the indicated Gal4 fusion protein expression plasmid as described previously (22).

U937 cells were maintained in spinner flasks containing RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and 5% CO₂. Differentiation of U937 cells was performed by adding all-*trans*-retinoic acid (Sigma) to a final concentration of 1 μM. Cells were then incubated for an additional 5 days prior to harvesting; cell cycle analysis of U937 cells was performed as described above for 3T3 cells (data not shown).

Plasmids—The plasmid GST-Myc-(1–143) contains the sequence encoding the first 143 amino acids of human c-Myc in the plasmid pGEX-4T-1 (Amersham Bioscience, Inc.). The human c-Myc sequence was obtained by PCR from the plasmid Gal4-Myc-(1–262), a gift from C. Dang, using the 5′ primer, 5′-CATAGAATAAGTGCACATCATCATC-GG-3′ and the 3′ primer, 5′-GGATCCTCGAGCTTGGCGGCGCCGA-GAA-3′. PCR products from the GST-Myc-(1–143) primers were isolated and digested with *Eco*RI and *Xho*I and ligated into *Eco*RI/*Xho*I digested pGEX-4T-1. The plasmid GST-USF-(1–181) contains the sequence encoding the first 181 amino acids of human USF1. The USF sequence was obtained from the plasmid Gal4-USF1, a gift from M. Eilers (37). The plasmid Gal4-USF1 was cut with *Eco*RI, and the insert was gel purified and ligated into *Eco*RI-digested pGEX-4T-1. Correct orientation of GST-Myc-(1–143) and GST-USF-(1–181) was confirmed by sequencing. The plasmids CMV-cdk9, CMV-cyclin T1, Gal4-cdk9, and Gal4-cyclin T1 were gifts from Luigi Lania (38). The plasmid Gal4-CBP is a gift from John Chrvia (39). The plasmid *cad* 3G4luc was constructed by the same method as *cad*4luc (22), but contains three Gal4 sites in tandem.

Measurement of RNA Levels—RNA was prepared from NIH 3T3 and U937 cells as described previously (33). RNase protections for *cad* mRNA levels in NIH 3T3 cells was performed as described previously (22, 40). RT-PCR was performed using the 5′ primer, 5′-CTCACT-GATCCCTCCTACAA-3′, and the 3′ primer, 5′-GTGGATACGACACT-GGGATA-3′, to amplify human *cad* mRNA. The mRNA from the human *gapdh* gene was detected using the 5′ primer, 5′-GAGCCAAAAGGGT-CATC-3′, and the 3′ primer, 5′-GTGGTCATGAGTCCCTC-3′. RT-PCR reactions contained 10 μl of EZ buffer (PerkinElmer Life Sciences), 2 μM of each primer, 1 M betaine (Sigma), 200 μM each dNTP, 5 units of rTth polymerase (PerkinElmer Life Sciences), 5 μl of 25 mM Mn(OAc)₂, and 300 ng of RNA in 50 μl total volume.

Formaldehyde Cross-linking and Chromatin Immunoprecipitation—The formaldehyde cross-linking and chromatin immunoprecipitation assays of tissue culture cells were performed as described previously with the following modifications (22, 41). Immunoprecipitations were performed overnight at 4 °C using 1 μg of anti-c-Myc sc-764-X (Santa Cruz), 1 μl of anti-USF1 serum (a gift from Emery Bresnick), anti-pol II sc-899 (Santa Cruz), or 3–5 μl of anti-pol II phospho-CTD serum (a gift from David Bentley). For the ChIP experiments with the anti-phospho-CTD antibody, 10 mM sodium pyrophosphate (pH 8) was added to all solutions to inhibit phosphatase activity. Before the first wash, 20% of the supernatant from the no antibody IP for each condition was saved as total input chromatin and was processed with the eluted IPs beginning with the cross-linking reversal step. After the final ethanol pre-

² M. A. Nikiforov, S. Chandriani, J. Park, I. Kotenko, D. Matheos, A. Johansson, S. B. McMahon, and M. D. Cole, submitted for publication.

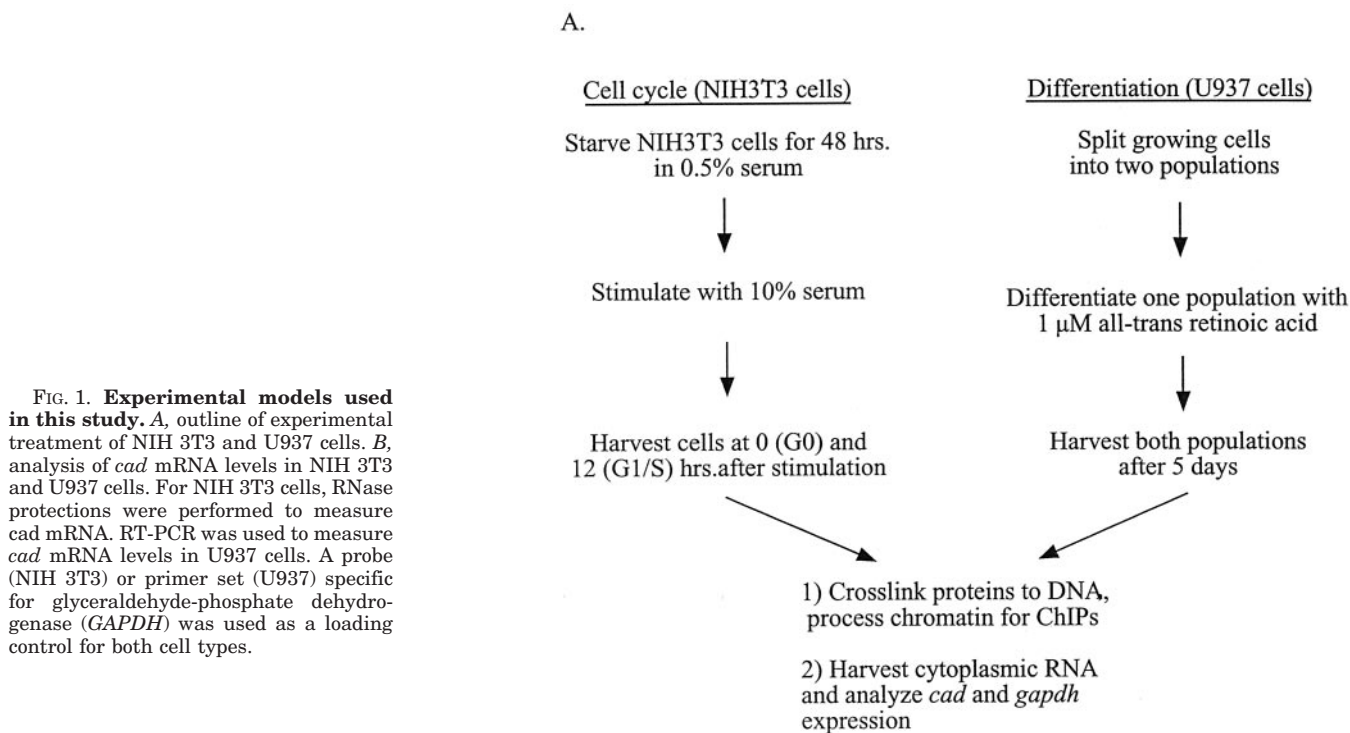
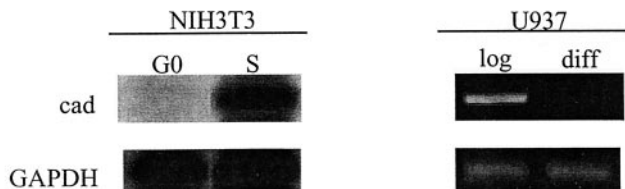


FIG. 1. **Experimental models used in this study.** A, outline of experimental treatment of NIH 3T3 and U937 cells. B, analysis of *cad* mRNA levels in NIH 3T3 and U937 cells. For NIH 3T3 cells, RNase protections were performed to measure *cad* mRNA. RT-PCR was used to measure *cad* mRNA levels in U937 cells. A probe (NIH 3T3) or primer set (U937) specific for glyceraldehyde-phosphate dehydrogenase (*GAPDH*) was used as a loading control for both cell types.

B.



precipitation, each IP sample was resuspended in 30 μl of PCR-grade TE (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, pH 8.0). Total input chromatin samples were resuspended in 30 μl of TE and further diluted 1/100 (total dilution 1/500).

PCR reactions contained 2 μl of IP sample or 2 μl of diluted total input (0.2% total input chromatin), 1.2 mM MgCl₂, 50 ng of each primer, 200 μM each dATP, dGTP, dCTP, and dTTP, 4 μl of 5 M betaine (Sigma), 1 × Thermophilic buffer (Promega), and 1.25 units of *Taq* DNA polymerase (Promega) in 20 μl total volume. After 34–36 cycles of amplification, 8 μl of the PCR products was electrophoresed on a 1.5% agarose gel, and DNA was stained with ethidium bromide and visualized under UV light. Alternatively, reactions were performed by incorporating ~2 μCi of [³²P]dCTP into the reactions and amplifying for 25 cycles. These products were separated on a 6% polyacrylamide gel and visualized with a Storm PhosphorImager (Molecular Dynamics). Signals were quantitated using ImageQuant for Macintosh v2.1 (Molecular Dynamics). Fold changes in factor binding were calculated by first dividing the IP signal intensity by the 0.2% input signal intensity (to control for varying levels of chromatin in each sample) for both the G₁/S (or log) sample and the G₀ (or differentiated) sample. Then, the G₁/S ratio was divided by the G₀ ratio. The sequences of primers used for PCR analysis can be found at our website (mcardle.oncology.wisc.edu/farnham).

Protein Affinity Chromatography—HeLa cell nuclear extract was prepared from frozen cells as described previously (42), and was dialyzed against affinity chromatography buffer (ACB) (10 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol) containing 0.5 mM phenylmethylsulfonyl fluoride and 0.1 M NaCl. GST fusion proteins were prepared and immobilized on glutathione-Sepharose 4B (Amersham Bioscience, Inc.), incubated with HeLa nuclear extract and columns were washed and proteins eluted as described previously (43). Eluates were analyzed by Western blot analysis with anti-cdk7 sc-529 (Santa Cruz), anti-cyclin H sc-855 (Santa Cruz), anti-cdk8 sc-1521 (Santa Cruz), anti-cyclin C sc-1061 (Santa Cruz), anti-cdk9 sc-7331

(Santa Cruz), and anti-cyclin T1 sc-8128 (Santa Cruz) antibodies. Each binding experiment was repeated at least three times.

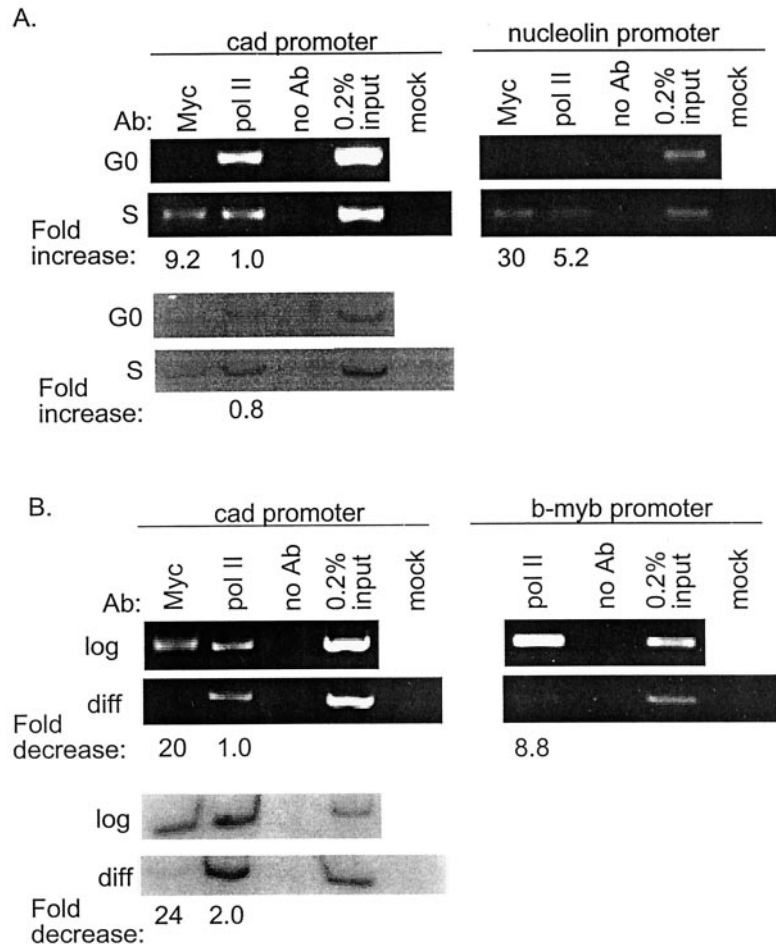
RESULTS

RNA Polymerase II Is Constitutively Bound to the *cad* Promoter—We had previously shown that there are high levels of acetylated histones on the *cad* promoter in both quiescent and differentiated cells (33) suggesting that the chromatin encompassing the *cad* promoter is in an open configuration prior to *c-Myc* binding. If so, components of the transcription machinery may have access to the *cad* promoter regardless of promoter activity. We therefore wished to examine the levels of RNAP II on the *cad* promoter in states of both high and low transcriptional activity. To do this we used both NIH 3T3 and U937 cells, so that we could monitor protein binding to *cad* under conditions where transcription can be regulated (Fig. 1). NIH 3T3 cells can be synchronized by serum withdrawal into quiescence, resulting in low levels of *cad* transcription. Addition of serum causes re-entry into the cell cycle, and *cad* transcription is maximal at S phase (Fig. 1B). U937 cells are a lymphoma-derived cell line that can be induced to differentiate after incubation with all-*trans*-retinoic acid. In growing U937 cells, *cad* expression is high, while differentiated cells show almost no *cad* mRNA (Fig. 1B).

To monitor RNAP II binding to the *cad* promoter, we used an antibody that recognizes the amino terminus of the large subunit of RNAP II. As a positive control, we also examined binding of *c-Myc* to the *cad* promoter. Immunoprecipitated DNA was analyzed by PCR using both ethidium bromide staining of

FIG. 2. RNA polymerase II is constitutively bound to the *cad* promoter.

A, ChIP experiment with NIH 3T3 cells. Formaldehyde cross-linked chromatin was isolated from cells that were synchronized by serum withdrawal and harvested at G₀ and S phases. Chromatin was immunoprecipitated with antibodies to c-Myc and the large subunit of RNAP II and analyzed by PCR using primers specific for the *cad* promoter and *nucleolin* gene. Below the *cad* promoter PCR is another PCR using the same primers but for only 25 cycles with incorporation of [α -³²P]dCTP. Fold increase in protein binding was calculated as detailed under "Materials and Methods." B, ChIP experiment in U937 cells. Asynchronously growing cells and cells treated with 1 μ M all-*trans*-retinoic acid for 5 days were harvested and processed for ChIPs as were NIH 3T3 cells. In addition to examining the *cad* promoter, PCR using primers which amplify the *b-myb* promoter was also performed. Below the *cad* promoter PCR is another PCR performed as in A.



34 cycle PCR and [α -³²P]dCTP incorporation of 25 cycle PCR. Both methods showed similar changes in protein binding (Figs. 2 and 3), so we have used ethidium bromide staining of 34 cycle PCR for the remainder of this study. As expected, we saw differences in the amount of c-Myc on the promoter which correlated with transcriptional activity. However, we found that levels of RNAP II did not correlate with transcriptional activity. Although c-Myc binding on the *cad* promoter increased 9.2-fold in NIH 3T3 cells from quiescence to S phase, RNAP II binding was unchanged (Fig. 2A). We also examined RNAP II binding on intron 1 of the *nucleolin* gene, which contains five E boxes which regulate *nucleolin* expression, and shows large changes in histone acetylation correlating with c-Myc binding (30). In contrast to the *cad* promoter which showed no difference in RNAP II binding, this region of the *nucleolin* gene shows a 5.2-fold change in RNAP II binding. Using the second model system, we saw a 20-fold difference in c-Myc binding on the *cad* promoter between growing and differentiated U937 cells, but RNAP II binding was unchanged in these cells (Fig. 2B). We also examined another cell cycle-regulated promoter, *b-myb*, for RNAP II binding in U937 cells. In contrast to the *cad* promoter, we saw an 8.8-fold difference in RNAP II binding to the *b-myb* promoter between growing and differentiated cells (Fig. 2B). The results obtained by monitoring the *nucleolin* gene and the *b-myb* promoter indicate that our assay is capable of detecting changes in RNAP II binding. Therefore, we conclude that RNAP II is bound to the *cad* promoter in quiescent NIH3T3 cells and differentiated U937 cells when *cad* transcription is low.

Although we see high levels of RNAP II on the *cad* promoter at all times, one would expect to see changes in the amount of

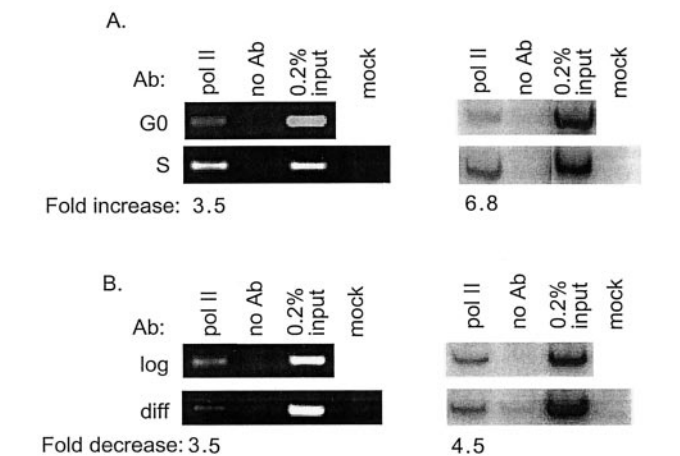


FIG. 3. Increased RNA polymerase II is seen at the 3' end of the *cad* gene when transcription is activated. A, NIH 3T3 cells synchronized at G₀ and S phase were cross-linked and chromatin was immunoprecipitated with a RNAP II antibody. Immunoprecipitated DNA was analyzed by PCR amplification with primers specific for the 3' end of the *cad* gene. On the left is a PCR reaction done for 34 cycles and stained with ethidium bromide and on the right is the same PCR done for 25 cycles with [α -³²P]dCTP incorporation. PCR signals were quantitated as detailed under "Materials and Methods." B, asynchronously growing U937 cells and U937 cells differentiated for 5 days with 1 μ M all-*trans*-retinoic acid were cross-linked and chromatin was immunoprecipitated with a RNAP II antibody. Immunoprecipitated DNA was analyzed by PCR as in A.

RNAP II on the coding regions of the *cad* gene correlating with changes in transcription. To test this hypothesis, DNA isolated from ChIP experiments was PCR amplified with primers to the

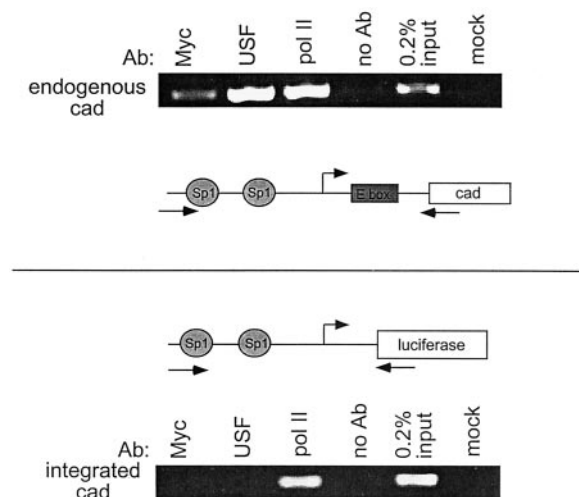


FIG. 4. The E box is not required to recruit RNA polymerase II to the *cad* promoter. A ChIP experiment was performed using NIH 3T3 cells stably transfected with a luciferase construct driven by the *cad* promoter containing sequences -81 to $+26$ relative to the transcription start site, which lacks the E box. Cross-linked chromatin was immunoprecipitated with antibodies recognizing *c-Myc*, USF, and RNAP II, and analyzed by PCR with primers specific for the endogenous and integrated *cad* promoters. Schematics of the endogenous and integrated promoters are shown between the PCR figures. Arrows indicate the relative location of the primers used for each PCR.

3' end of the *cad* gene. We found that RNAP II binding changes about 3–5-fold on the 3' end of *cad* in NIH 3T3 cells between G_0 and S phase or as growing U937 cells are differentiated (Fig. 3, A and B). We have repeated the ChIP assay several times and have obtained similar results. These results suggest that *c-Myc* may indeed activate transcription of *cad* by stimulating the transition between the initiation complex and the elongation complex, thus resulting in an increase in full-length transcripts.

Our results clearly indicate that RNAP II is bound to the *cad* promoter in the absence of *c-Myc*. These results raise the question as to how RNAP II is recruited to the *cad* promoter. The *cad* promoter does not have a TATA box so recruitment of RNAP II by TFIID might not be the mechanism by which RNAP II is brought to the promoter. We have previously shown that USF is bound to the *cad* promoter in quiescent NIH3T3 cells, when *cad* transcription is very low (22). Although USF does not activate the *cad* promoter, it is possible that USF binding to the *cad* promoter may be required to recruit RNAP II and a switch between USF and Myc/Max may then allow transcription to begin. To test this possibility, ChIPs were performed on a cell line that contains a stably integrated luciferase reporter driven by a derivative of the *cad* promoter which lacks an E box. We had previously shown that this integrated promoter could not recruit Myc or USF and that the promoter did not show an increase in activity upon serum stimulation (21, 41). However, previous studies had not examined the effect of deletion of the E box on RNA polymerase II recruitment. As a control, we first monitored binding of *c-Myc*, USF, and RNAP II on the endogenous *cad* promoter. As expected, we found that all three antibodies immunoprecipitated the endogenous *cad* promoter. Furthermore, we found that the integrated promoter lacking an E box is not bound by either *c-Myc* or USF, confirming our previous studies which indicated that the E box was necessary for binding of both helix-loop-helix leucine zipper proteins (41). When levels of RNAP II were examined on the mutated *cad* promoter, we found that RNAP II was bound to the mutated *cad* promoter despite the absence of *c-Myc* and USF, at similar levels found on the endogenous *cad* promoter

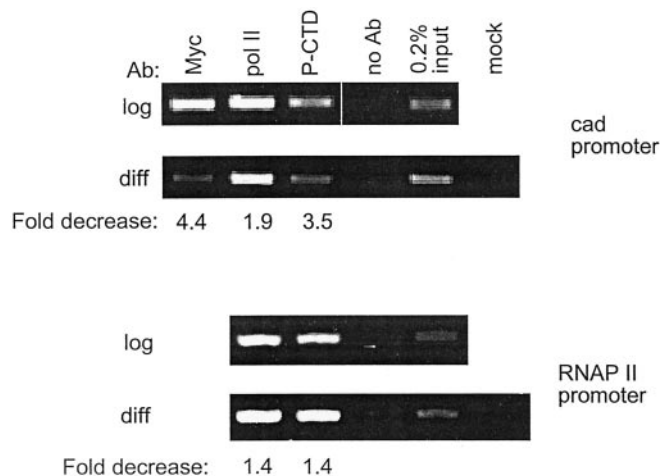


FIG. 5. Decreased phosphorylation of the RNA polymerase II CTD on the *cad* promoter in differentiated U937 cells. Asynchronously growing and differentiated U937 cells were cross-linked and chromatin was precipitated with antibodies to Myc, RNAP II (NH₂ terminus), and the hyperphosphorylated form of the large subunit CTD. Immunoprecipitated DNA was analyzed by PCR using primers specific to the *cad* promoter and the *RNA polymerase II large subunit* promoter, and PCR signals were quantitated as described under “Materials and Methods.”

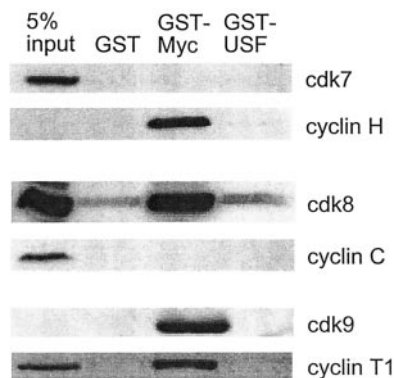
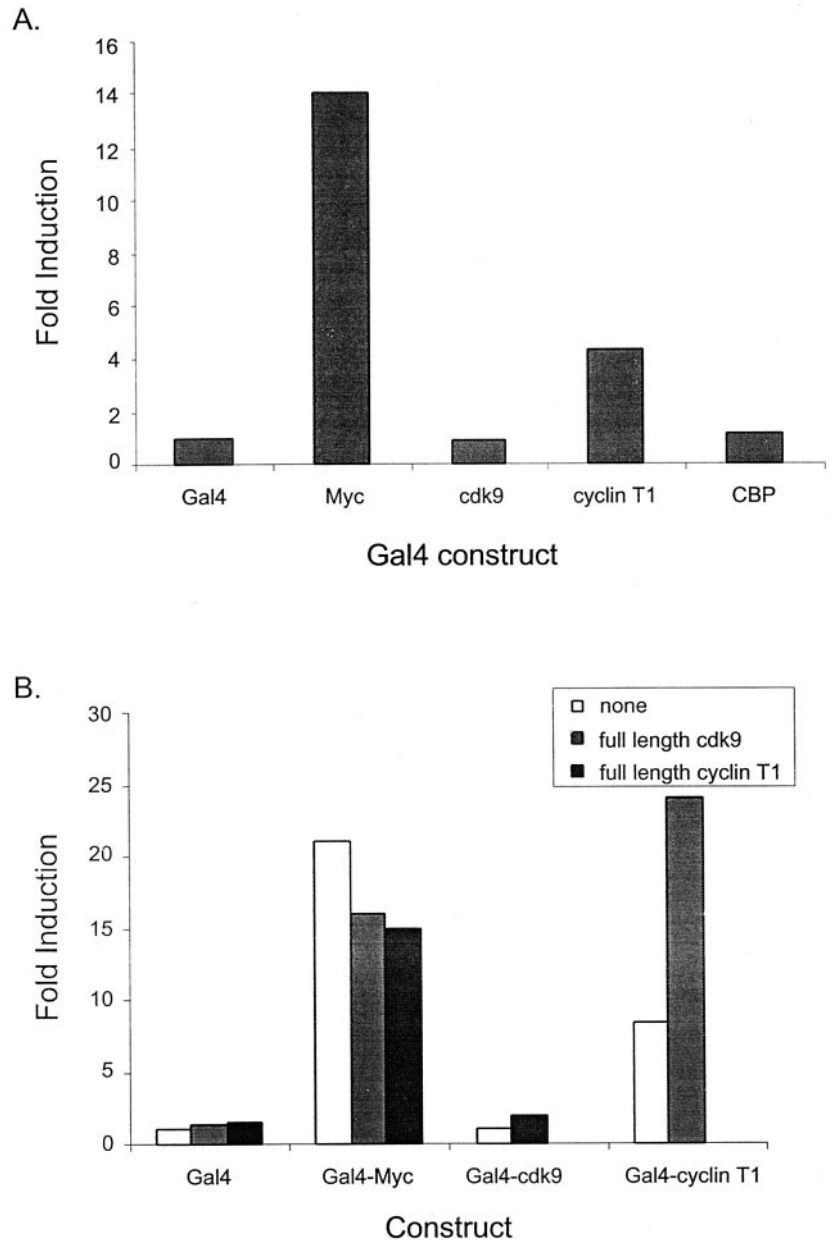


FIG. 6. The *c-Myc* transactivation domain interacts with P-TEFb. GST fusions of the *c-Myc* and USF transactivation domains were immobilized on a glutathione column and HeLa nuclear extract was poured over the column. After washing the column, proteins were eluted and analyzed by Western blot using antibodies for cdk7, cyclin H, cdk8, cyclin C, cdk9, and cyclin T1.

(Fig. 4). These results indicate that the E box is not required for recruitment of RNAP II to the *cad* promoter. The integrated construct contains the two Sp1 sites found in the wild type promoter (Fig. 4). DNA footprinting studies have shown that Sp1 and a ubiquitous protein called Honk are the only proteins that bind to the region of the *cad* promoter contained in our integrated promoter, and only Sp1 affects transcription of *cad* (44). Thus, it is likely that Sp1 recruits RNAP II to the promoter. Our finding that the E box in the *cad* promoter is not required to recruit RNAP II is consistent with our experiments showing that RNAP II is bound to the *cad* promoter prior to binding of *c-Myc*, supporting the hypothesis that *c-Myc* may activate transcription by stimulating promoter clearance.

Phosphorylation of the RNAP II COOH-terminal Domain Correlates with *c-Myc* Binding—The results from our ChIP assay on binding of RNAP II to the *cad* promoter and coding regions imply that *c-Myc* may function to stimulate elongation. It is known that in order for efficient elongation of mRNA to occur, the CTD of the large subunit of RNAP II must be hyperphosphorylated (45). Thus, if *c-Myc* acts by stimulating elongation, the presence of the hyperphosphorylated form of the pol

FIG. 7. Cyclin T1 plus cdk9 can substitute for c-Myc in activation of the *cad* promoter. A, asynchronously growing NIH 3T3 cells were co-transfected with 0.5 μ g of *cad* 3G4luc, and 2.5 μ g of the indicated Gal4 construct. Cells were harvested and lysates were analyzed for luciferase activity. *Fold induction* refers to the ratio of the Gal4 fusion construct signal to that of the Gal4 vector. B, repeat of experiment shown in A. In addition, 1 μ g of plasmids containing the CMV promoter driving expression of full-length cdk9 or cyclin T1 were co-transfected with *cad* 3G4luc and the Gal4 constructs as indicated.

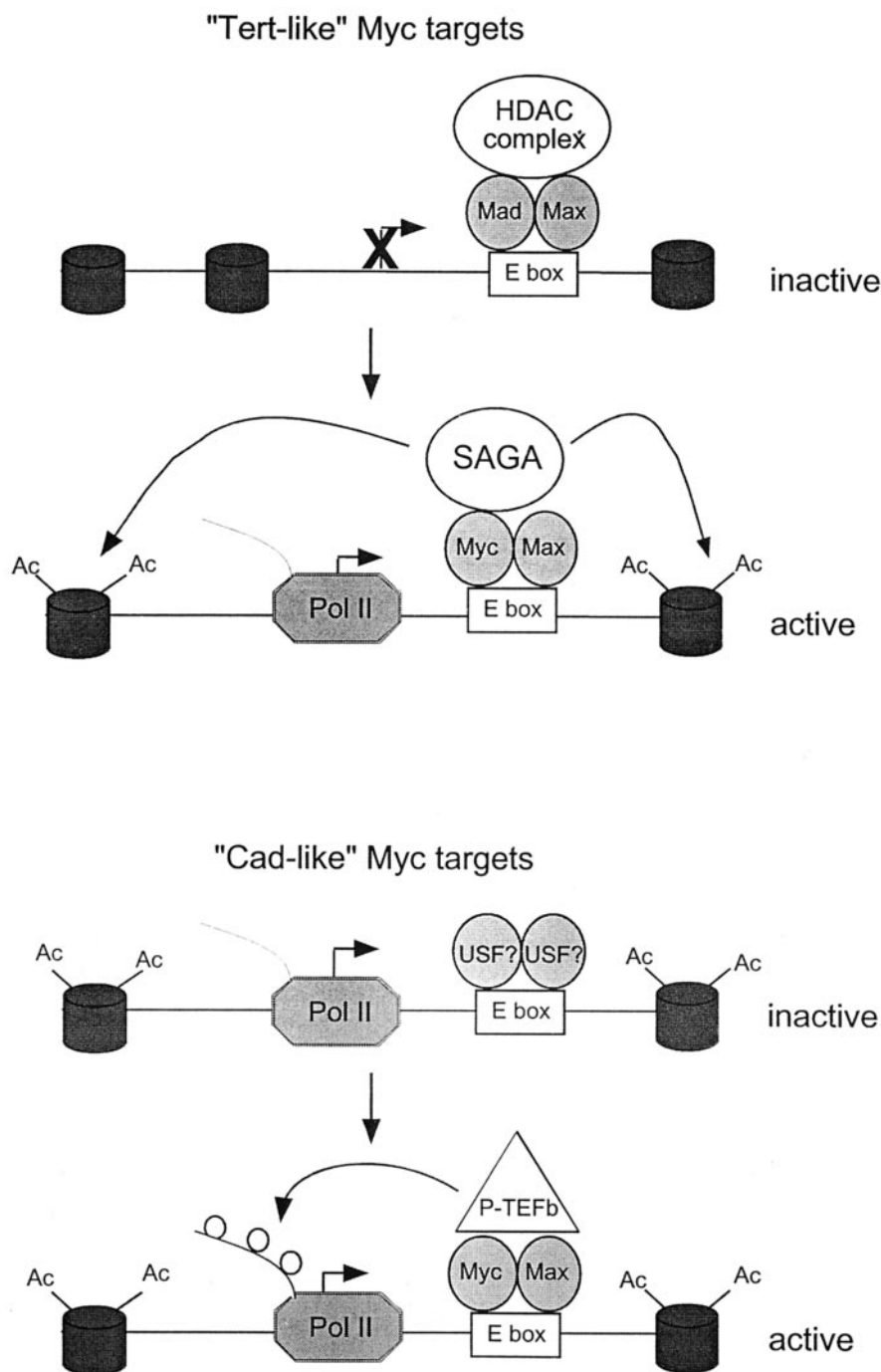


II CTD should coincide with c-Myc binding to the *cad* promoter. To test this hypothesis, we performed the ChIP assay on asynchronously growing and differentiated U937 cells using an antibody that was raised against a hyperphosphorylated RNAP II CTD peptide. Crude serum from a rabbit immunized with this peptide recognizes both the hyperphosphorylated and hypophosphorylated forms of RNAP II (46). However, passing the serum over an unphosphorylated CTD peptide column depletes the serum of antibodies that recognize the hypophosphorylated form of the CTD, thus enriching for antibodies which recognize the hyperphosphorylated CTD (46). Using this "enriched" serum, we found that the levels of phosphorylated CTD on the *cad* promoter closely correlate with the change in c-Myc binding seen as U937 cells differentiate (Fig. 5). Although we did see higher levels of phosphorylated RNAP II CTD on the *cad* promoter in growing *versus* differentiated cells, the difference was not large. This could be due to the fact that, once phosphorylated, the RNAP II immediately begins elongating and vacates the promoter region. Also, the phospho-CTD antibody used in this experiment only recognizes Ser-5 of the CTD heptapeptide repeat and it has been shown that Ser-5 of the repeat

is only transiently phosphorylated on the promoter (47). In addition, we examined the *RNAP II* promoter, which is constitutively transcribed in U937 cells (48). On this promoter both the antibody against the NH₂-terminal domain of the RNAP II large subunit and the anti-phospho-CTD antibody showed only slight decreases in binding, demonstrating that the decrease in the amount of phosphorylated RNAP II on the *cad* promoter is not due to loss of chromatin or the presence of phosphatase activity during chromatin preparation. We also performed a ChIP experiment with the phospho-CTD antibody on synchronized NIH 3T3 cells. However, the antibody was unable to immunoprecipitate any DNA in these cells, possibly because the epitope that the antibody recognizes is somehow masked (data not shown).

We have also attempted ChIPs with the commercially available H5 and H14 antibodies that recognize phosphorylated Ser-2 and Ser-5 of the RNAP II CTD heptapeptide repeat, respectively. These antibodies have been used for ChIPs in other studies to examine the phosphorylation status of the RNAP II CTD on a number of genes (47, 49). Using these antibodies the *cad* promoter was immunoprecipitated from

FIG. 8. Possible mechanisms of transcriptional activation by *c-Myc*. *c-Myc* target genes may fall into two categories, "Tert-like" genes and "Cad-like" genes. Tert-like genes, such as *tert*, *cyclin D2*, and *nucleolin*, have Mad/Max bound to their E boxes when the genes are turned off, and have deacetylated histones due to the action of histone deacetylases brought in by Mad. When Mad/Max heterodimers are replaced by Myc/Max, Myc recruits the SAGA complex through interaction with TRRAP, and RNAP II is recruited to the promoter and transcription begins. Alternatively, Cad-like genes have constitutively acetylated histones and bound RNAP II which cannot elongate due to a hypophosphorylated CTD. Recruitment of P-TEFb by Myc/Max heterodimers bound to target promoters stimulates CTD phosphorylation and facilitates promoter clearance and elongation. Note that Tert-like genes may also require recruitment of P-TEFb by Myc to achieve full transcriptional activation.



both growing and differentiated U937 cells (data not shown). Although these results might be interpreted to suggest that the *cad* gene is transcribed constitutively, this conclusion does not agree with many previous studies showing cell cycle regulation of the *cad* promoter. Another possibility is that the H5 and H14 antibodies are immunoprecipitating genes that are inactive. To test this possibility we performed PCR on immunoprecipitated chromatin with primers to the α -fetoprotein promoter, which is inactive in U937 cells. We found a significant signal with both the H5 and H14 antibodies. In contrast, an antibody which recognizes the amino-terminal domain of the RNAP II large subunit failed to immunoprecipitate the α -fetoprotein promoter (data not shown). Thus it appears that the H5 and H14 antibodies may be recognizing proteins other than the phosphorylated CTD, and so the ChIPs results from these antibodies for the *cad* promoter cannot be interpreted.

***Myc* Interacts with the CTD Kinase Complex P-TEFb**—Because phosphorylation of the RNA pol II CTD coincides with *c-Myc* binding to the *cad* promoter, we reasoned that *c-Myc* may stimulate elongation by recruiting a kinase that phosphorylates the CTD. Also, the failure of USF to activate the *cad* promoter (41) may be due to its inability to recruit the same kinase. Although many kinases have the ability to phosphorylate the CTD, the cyclin-dependent kinases *cdk7*, *cdk8*, and *cdk9* have been most strongly implicated in stimulating elongation. Therefore, we wished to determine if *c-Myc* interacts with any of these CTD kinases and/or their respective cyclins using a GST pull down assay. Constructs were made in which the *c-Myc* and USF1 TADs (amino acids 1–143 of *c-Myc* and amino acids 1–181 of USF1) were fused to GST. Although we subsequently use amino acids 1–262 of *c-Myc* for transcriptions studies (Fig. 7), we constructed the GST fusion with amino

acids 1–143, which has equivalent activation ability to 1–262 (9), to simplify future deletion analysis. The GST fusion proteins were bound to glutathione-Sepharose columns, HeLa nuclear extract was poured over the columns, and bound proteins were analyzed by Western blot. Western blots from these experiments were probed with antibodies recognizing cdk7/cyclin H, cdk8/cyclin C, and cdk9/cyclin T1. We found that while the USF1 TAD does not interact with any of these proteins, the c-Myc TAD recruits cdk8, cyclin H, cdk9, and cyclin T1 to the column (Fig. 6). We were unable to find any protein which binds to the USF1 TAD. At this time there are no proteins known to bind to the USF1 TAD. In fact, the USF proteins do not appear to be involved in many protein-protein interactions. For example, a recent study used USF1 as bait for a yeast two-hybrid experiment. The authors found that out of 4.8×10^6 transformants, only two positive clones were isolated, both containing the cDNA for Fra1 (50). Thus it is not surprising that we could detect no protein interactions with the USF TAD. Although cdk8 and cyclin H were recruited to the GST-Myc column, their respective partners, cyclin C and cdk7, were not. However, both cdk9 and cyclin T1 were pulled down by the Myc TAD and they do form a functional cyclin/cdk pair, *i.e.* P-TEFb. Because recruitment of both components of a complex are necessary for phosphorylation to occur, we have focused on the interaction between c-Myc and P-TEFb.

Cyclin T1 Activates the *cad* Promoter—We have demonstrated an interaction between the c-Myc transactivation domain and the P-TEFb components cdk9 and cyclin T1. We next wished to determine if cdk9 and/or cyclin T1 could substitute for c-Myc to activate the *cad* promoter. Therefore, we performed a transactivator bypass experiment in which Gal4-*cdk9*, Gal4-*cyclinT1*, and Gal4-*Myc*-(1–262) constructs were used in a co-transfection with *cad* 3G4luc, a luciferase reporter that is driven by the –81/+26 minimal *cad* promoter with three Gal4 sites substituted for the E box. The Gal4-Myc TAD construct activated transcription ~14-fold, in agreement with previous results (22). In contrast, Gal4-*cdk9* did not activate *cad* 3G4luc, while the Gal4-*cyclin T1* construct was able to activate *cad* 3G4luc ~4-fold (Fig. 7A). If P-TEFb were a necessary cofactor for Myc-mediated activation of *cad* transcription, then one might expect that either Gal4-*cdk9* or Gal4-*cyclin T1* would activate the *cad* promoter, yet the Gal4-*cdk9* construct was essentially nonfunctional. However, it is possible that this fusion protein does not function efficiently in the context of the *cad* promoter. To determine if all Gal4 fusion proteins would activate the *cad* promoter, we have used Gal4-CBP. We have previously shown that Gal4-CBP can activate the *dhfr* promoter (43). However, Gal4-CBP only activated *cad* 3G4luc 1.4-fold more than the Gal4 vector. Therefore, not all Gal4 transactivator fusion proteins can stimulate the *cad* promoter.

Because cdk9 requires cyclin T1 for its function and vice versa, we hypothesized that levels of endogenous cdk9 and cyclin T1 may be limiting for the Gal4-*cdk9* and Gal4-*cyclin T1* constructs' function, resulting in low activation of the *cad* promoter. Therefore, constructs expressing full-length cdk9 and cyclin T1 driven by the CMV promoter were transfected into cells along with the Gal4 constructs. Co-transfection of cdk9 and cyclin T1 had little effect on the Gal4 vector, and a slight inhibitory effect on Gal4-Myc was seen. Co-transfection of cyclin T1 with Gal4-*cdk9* did not result in activation of *cad* 3G4luc. It remains possible that the conformation of the Gal4-*cdk9* fusion is not optimal for activation of *cad*. However, expression of full-length cdk9 along with Gal4-*cyclin T1* enhanced activation of *cad* 3G4luc to levels equivalent to that of Gal4-Myc (Fig. 7B). These results suggest that Myc activates *cad* transcription through recruitment of P-TEFb to the promoter.

Although the Gal4-*cyclin T1* experiments indicate that cyclin

T1 can substitute for Myc in mediating transcription of the *cad* promoter, they are not conclusive proof that Myc does in fact recruit cyclin T1 to the endogenous *cad* promoter. Better evidence would be provided by the demonstration that an antibody to cyclin T1 could immunoprecipitate the *cad* promoter in the presence, but not the absence, of bound c-Myc. Unfortunately, we have tried various antibodies against cyclin T1, all of which have been unsuccessful in the chromatin immunoprecipitation assay (data not shown). Control experiments have indicated the failure of these antibodies to immunoprecipitate the *cad* promoter is due to technical, not biological, reasons. Perhaps more robust antibodies will allow cyclin T1-specific chromatin immunoprecipitation to be performed in the future. However, the fact that Myc binding and RNA polymerase phosphorylation correlate with *cad* transcription, the fact that we can show an interaction between Myc and cyclin T1 *in vitro*, and the fact that Gal4-*cyclin T1* can substitute for Gal4-Myc taken together supports the hypothesis that Myc activates the *cad* promoter via recruitment of cyclin T1.

DISCUSSION

We have previously shown that transcriptional activation of the *cad* promoter correlates with binding of c-Myc to an E box located 65 base pairs downstream of the transcription start site. Because the *cad* promoter exists in an open chromatin conformation prior to c-Myc binding, as monitored by the presence of bound RNA polymerase II, we suggest that Myc activates transcription by stimulating productive transcriptional elongation. We also show that c-Myc can interact with the components of the elongation factor P-TEFb and that one component of P-TEFb, cyclin T1, can stimulate transcription from the *cad* promoter in a transactivator bypass experiment. Thus, the rate-limiting step of transcriptional activation of the *cad* promoter may be Myc-mediated recruitment of P-TEFb, which then allows phosphorylation of RNAP II and release of a poised transcription complex.

Our data indicate that RNAP II complexes are bound to the *cad* promoter regardless of transcriptional activity. Others have previously shown that promoters such as *c-fos*, *c-myc*, and *hsp70* are also bound by stalled, or paused, RNA polymerases (51–53). In fact, one study suggests that 21% of the detectable transcribing RNAP II complexes are paused (54). Productive elongation is thought to be regulated by the cyclin-dependent kinase, P-TEFb, which has been shown to phosphorylate the CTD of RNAP II (55). Artificial targeting experiments have demonstrated that recruitment of components of P-TEFb can activate gene expression. For example, Gal4-*cdk9* and Gal4-*cyclin T1* can activate a *hsp70*-based promoter reporter and some, but not all, artificial promoters (38, 56). However, few studies have revealed the mechanism by which P-TEFb finds a target promoter. The best characterized system is the HIV long terminal repeat. Several studies have shown that the viral transactivator Tat can interact with cdk9/cyclin T1, thus recruiting P-TEFb to the TAR element located just downstream of the HIV start site (57, 58).

Other cellular transcription factors have been proposed to recruit P-TEFb to a natural promoter. One is CIITA, a co-activator of major histocompatibility complex class II genes. It has been proposed that P-TEFb is recruited to major histocompatibility complex class II promoters via CIITA, which in turn may be recruited to the DNA by interaction with DNA-binding proteins such as RFX and NFY (59). However, as of yet, there is no data demonstrating that RFX or NFY are the DNA-binding factors involved in P-TEFb recruitment. P-TEFb has also been shown to interact with the androgen receptor (60). A mutant form of cdk9 was able to inhibit an androgen receptor-regulated viral reporter gene, and transcription of this reporter

was inhibited by 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole, a purine nucleotide analog which inhibits P-TEFb function. However, no cellular promoter was shown to be regulated by androgen receptor-mediated recruitment of P-TEFb. Also, while our studies were in progress, Barboric *et al.* (61) reported that NF- κ B binds P-TEFb and recruits cdk9 and cyclin T1 to the interleukin-8 promoter. Myc is now another example of a site-specific DNA-binding transcription factor that can recruit P-TEFb. Interestingly, the location of the E box in the *cad* promoter (at +65) is similar to the location of the TAR element, suggesting that both Myc (a DNA-binding protein) and Tat (an RNA-binding protein) function in a similar manner to recruit P-TEFb to a location just downstream of a paused RNAP II transcription complex. We suggest that the inability of USF to activate *cad* transcription is due to the inability of USF to recruit P-TEFb (Fig. 6). Although the mechanism by which USF functions as an activator is unclear it is known that transactivation of genes by USF is highly dependent on promoter context. For example, one study showed that activation by USF of an initiator-driven promoter was enhanced by inserting a TATA box (62). Thus, the *cis*-acting elements necessary for USF to activate transcription may not exist on the *cad* promoter. Also, transactivation by USF has been shown to be cell type-specific (63).

It has been proposed that there are three classes of transcriptional activation domains (64). Type I activators, such as Sp1 and CTF, can only stimulate initiation, most likely through recruitment of components of the general transcriptional machinery. Our data suggesting that Sp1 recruits RNAP II to the *cad* promoter supports the hypothesis that Sp1 functions at an early step in transcription initiation at the *cad* promoter. Type IIA activators, such as Tat, can only stimulate elongation. In the case of Tat, this stimulation is due to recruitment of P-TEFb. Finally, type IIB activators, which include VP16, p53, and E2F1, can stimulate both initiation and elongation. Factors such as VP16 and E2F1 can bind to both HATs and CTD kinases (34, 43, 65). Based on our results, we suggest that c-Myc is a type II activator. c-Myc shares characteristics with both IIA and IIB activators. Like Tat, c-Myc binds to P-TEFb and, on many of its target genes, binds downstream of the transcriptional start site. However, c-Myc also has the ability to recruit HAT activity (28). Therefore, c-Myc may be a type IIB activator with the relative importance of its two functions differing from promoter to promoter.

We suggest that c-Myc functions to regulate the *cad* promoter by stimulating promoter clearance (Fig. 8). The *cad* gene exhibits a low basal level of promoter activity that is increased in late G₁ upon accumulation of c-Myc protein. Extensive remodeling by histone deacetylases and acetylases in every cell cycle is not necessarily the most efficient mechanism of regulation for this cyclical expression pattern. Our studies showing that the *cad* promoter is occupied by RNAP II prior to Myc binding supports the hypothesis that, in quiescent and differentiated cells, the *cad* promoter is occupied by an inactive preinitiation complex. The cell cycle-dependent increase in binding of Myc to the *cad* promoter thus allows a rapid increase in productive transcription without the need for extensive chromatin remodeling or assembly of large preinitiation complexes. Although our data indicates that the ability of Myc to stimulate transcriptional elongation may be most important at the *cad* promoter, we also stress that c-Myc may play a key role in other steps in the transcription process at other promoters. For example, if a promoter does not already exist in an open chromatin conformation prior to c-Myc binding, then binding of c-Myc may alter histone acetylation, which then enhances polymerase recruitment (Fig. 8). Such promoters may include the *tert*,

cyclin D2, and *nucleolin* promoters (30–32). Our studies suggesting that there are at least two categories of Myc-regulated promoters are supported by the work of others which indicate that different regions of the Myc transactivation domain are required to activate different promoters. For example, overexpressed c-Myc lacking the TRRAP interaction domain retains the ability to activate the *cad* promoter,² supporting our data which suggests that a Myc function distinct from HAT recruitment is most important for activation of *cad* transcription. We propose that future studies of the mechanism(s) by which c-Myc activates transcription should focus on individual Myc target promoters to determine the step(s) of transcriptional activation Myc acts upon.

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