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Direct Examination of Histone Acetylation on Myc Target Genes Using Chromatin Immunoprecipitation*

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Overexpression of c-Myc can lead to altered transcriptional regulation of cellular genes and to neoplastic transformation. Although DNA binding is clearly required, the mechanism by which recruitment of c-Myc to target promoters results in transcriptional activation is highly debated. Much of this controversy comes from the difficulty in clearly defining a true Myc target gene. We have previously determined that *cad* is a *bona fide* Myc target gene and thus now use the *cad* promoter as a model to study Myc function. Others have shown that Myc can interact indirectly with histone acetylases and have suggested that Myc mediates transcriptional activation by causing an increase in the levels of acetylated histones on target promoters. To directly test this model, we employed a chromatin immunoprecipitation assay to examine the levels of acetylated histones on the *cad* promoter. Although Myc was bound to the *cad* promoter in S phase but not in G_0 phase, we found high levels of acetylated histones on the promoter in both stages. We also examined acetylated histones on the *cad* promoter before and after differentiation of U937 cells. Although the levels of c-Myc bound to the *cad* promoter were greatly reduced after differentiation, we saw high levels of acetylated histones on the *cad* promoter both before and after differentiation. Finally, we found that a 30-fold change in binding of N-Myc to the telomerase promoter did not result in a concomitant change in histone acetylation. Thus, recruitment of a Myc family member to a target promoter does not necessarily influence the amount of acetylated histones at that promoter. Further investigations are in progress to define the role of Myc in transcriptional activation.

The c-Myc oncoprotein has been found to be deregulated in many different types of cancer, including breast, colon, and prostate cancers, as well as many types of leukemias and lymphomas. It is overexpressed in tumors by many different mechanisms, including gene amplification, translocation, retroviral insertion, and other means (1). The role of c-Myc in cell proliferation has been documented by numerous studies. Increased expression of c-Myc is thought to be an important contributor to the neoplastic transformation of the tumor based on intentional overexpression of c-Myc in tissue culture cells and in transgenic mice. For example, cotransfection of Myc and Ras in rat embryo fibroblasts causes these cells to adopt a transformed phenotype (2). Overexpression of c-Myc in tissue culture causes increased proliferation of cells with a shortened G_1 phase, whereas loss of c-Myc results in slow growth and a longer G_1 phase (3). Finally, studies of transgenic mice in which c-Myc overexpression is targeted to a specific tissue have also demonstrated the importance of c-Myc in tumorigenesis. Targeted overexpression of c-Myc in the breast, B lymphocytes, and liver in transgenic mice results in increased formation of tumors in these tissues, although tumor formation is greatly enhanced by coexpression of a cooperating oncogene, such as Ha-Ras (4–7).

The c-*myc* gene encodes a protein that is a member of the basic/helix-loop-helix/leucine zipper family of transcription factors. Myc heterodimerizes with the protein Max through its C-terminal helix-loop-helix and leucine zipper domains and binds to an E box motif, which is the hexanucleotide sequence CACGTG (8). Myc/Max heterodimers activate transcription because of a transactivation domain in the N terminus of the Myc protein (9). Max is also a heterodimerization partner for the Mad proteins; however, Mad/Max heterodimers bind to E boxes and repress transcription via a transcriptional repression domain in the Mad protein (10).

A commonly accepted model of Myc-mediated transcriptional regulation invokes an exchange of co-repressors and co-activators on Myc target genes that occurs as Myc/Max heterodimers replace Mad/Max heterodimers (Fig. 1). Specifically, it is thought that binding of Mad/Max heterodimers to promoter DNA results in transcriptional repression via histone deacetylation because of the recruitment of mSin3A or mSin3B and subsequent recruitment of HDAC1 or HDAC2 (11, 12). These transcriptional repressor complexes are thought to bind to Myc target genes in quiescent or differentiated cells, *i.e.* in cells that contain very low amounts of Myc protein. However, proliferating cells contain increased amounts of Myc, and Myc/Max heterodimers replace Mad/Max heterodimers on target promoters. Because Myc can interact with TRRAP, which in turn interacts with the histone acetyltransferase hGCN5, it has been suggested that replacement of Mad/Max with Myc/Max results in increased amounts of acetylated histones on Myc target genes (13). It is believed that acetylation of lysines of the N-terminal tails of histones in core nucleosomes promotes chromatin remodeling, which then allows site specific transcription factors and the RNA polymerase II basal transcriptional machinery access to the start site of transcription (for review see Ref. 14).

A direct test of the model described above requires examination of promoter occupancy by acetylated histones on a Myc target gene. However, there is great deal of uncertainty as to which genes are actually regulated by c-Myc. A recent study found that only 5% of the 6000 genes tested using DNA microchips responded to overexpression of c-Myc (15). A number of

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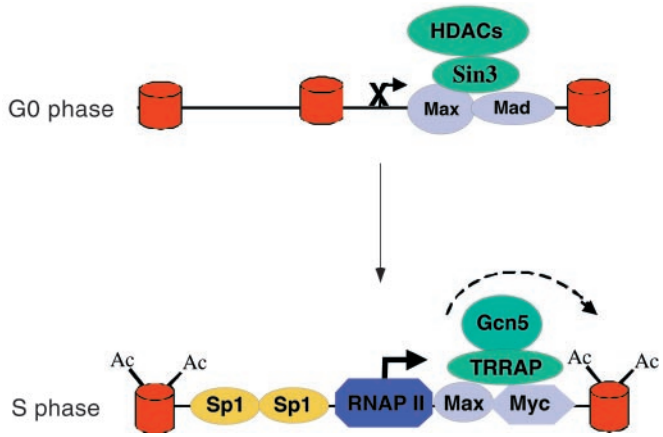


FIG. 1. **Current model for Myc-mediated transcriptional activation.** Transcriptional repression mediated by Mad/Max is believed to be due to recruitment of the mSin3 repressor complex. mSin3 binds to HDACs, which remove acetyl groups from histone tails and maintain chromatin in a repressive configuration. In contrast, Myc is thought to activate target gene transcription by recruiting proteins with histone acetyltransferase activity through TRRAP or other means. The addition of acetyl groups onto histone tails would result in a more open chromatin configuration allowing RNA polymerase II to bind the promoter and begin transcription.

candidate genes have been proposed to be targets of Myc regulation, including *odc* (ornithine decarboxylase) (16), the translation initiation factor *eIF4E* (17), the catalytic subunit of telomerase (*tert*) (18), and *cdk4* (19). Another complicating factor in the study of Myc target genes is the modest transcriptional activity mediated by the Myc protein. Most genes that respond to increased levels of c-Myc change by only 2–5-fold. Some of the difficulty in identifying a Myc target gene may be due to the presence of other, more abundant, proteins in the cell (such as USF) that can bind to E boxes. Therefore, identification of Myc target genes requires demonstrating that binding of Myc to the promoter region is required for transcriptional regulation.

We have recently shown a correlation between promoter occupancy by c-Myc and transcriptional activation of the *cad* (carbamoyl phosphate synthase/aspartate transcarbamoylase/dihydroorotase) gene. The *cad* gene encodes the trifunctional enzyme carbamoyl phosphate synthase/aspartate carbamoyltransferase/dihydroorotase, which catalyzes the first three rate-limiting steps of pyrimidine biosynthesis. *cad* expression is minimal in quiescent cells, and expression increases as cells enter the cell cycle, with peak expression occurring at the G_1/S phase boundary. The *cad* gene contains an E box downstream from the transcription start site that is conserved in the mouse, rat, hamster, and human homologues of the gene. Chromatin immunoprecipitation studies have shown that both Myc and USF bind to the *cad* promoter (20). However, mutations flanking the E box that disrupt Myc binding to the *cad* promoter but do not affect USF binding result in a loss of growth regulation (21). Taken together, these results suggest that *cad* is a *bona fide* Myc target gene, and thus in the studies described below we have used the *cad* promoter to study Myc function.

We have used a chromatin immunoprecipitation assay to determine whether changes in histone acetylation correlate with Myc-mediated changes in *cad* expression. We found only modest differences in histone acetylation on the *cad* promoter as NIH3T3 cells enter the cell cycle from quiescence or as U937 cells exit the cell cycle into a differentiated state. Furthermore, our studies indicate that high levels of histone acetylation are present at the *cad* promoter in G_0 and in S phase. This high degree of acetylation is not seen in the transcribed regions of the *cad* gene or at a promoter that is never active in NIH3T3 cells. Additionally, we have examined an N-Myc responsive promoter in neuroblastoma cells, and we do not see changes in histone acetylation that correlate with N-Myc binding. Our results suggest that although high levels of histone acetylation can identify a promoter that has transcriptional potential in a certain cell type, histone acetylation does not appear to be the mechanism by which Myc regulates transcription.

MATERIALS AND METHODS

Cell Culture—NIH3T3 cell cultures were maintained and synchronized as described previously (22). Briefly, $6-8 \times 10^6$ cells were seeded into 500-cm^2 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 G_1 phase) or 12 h (G_1/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 (23). Data were acquired on a FACScan flow cytometer (Becton Dickinson) using CellQuest acquisition and analysis software. The treatment of G_0 phase cells with trichostatin A (TSA)[1] was performed as follows. 4×10^6 cells were seeded into 225-cm^2 tissue culture flasks (5 flasks/experimental condition; combined prior to cell lysis) and incubated in starvation medium for 48–52 h to induce quiescence. Trichostatin A (Sigma) (1 mg/ml, solubilized in 100% ethanol) was diluted in medium and added to the cells at a final concentration of 1 μg/ml of medium (3.30 μM). Cells were then incubated for an additional 16–20 h prior to harvesting. Untreated cells received an equal volume of ethanol without TSA, whereas S phase cells received only stimulation medium 12 h prior to harvesting of RNA and protein.

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% CO_2. 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.

Tet21N cells were maintained at 37 °C and 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 4 mM L-glutamine. Tetracycline was added to a final concentration of 1 μg/ml for 2 weeks to repress N-Myc transcription.

RNase Protection Assay—RNA preparation and RNase protection assays were performed as described previously (20, 24). For analysis of *cad* mRNA, 35–40 μg of cytoplasmic RNA from serum-starved, serum-stimulated, or TSA-treated cells was incubated with 1×10^5 cpm of probe at 65 °C for 3 h. For analysis of glyceraldehyde-phosphate dehydrogenase mRNA, 5–10 μg of cytoplasmic RNA was incubated with 1.7×10^4 cpm of probe at 52 °C for 3 h. Unhybridized RNA was digested by the addition of 10 μg of RNase A. The products were resolved on an 8% denaturing polyacrylamide gel and visualized by autoradiography. Signals were collected using a PhosphorImager and quantitated using ImageQuant v4.2a (Molecular Dynamics).

Acid-soluble Nuclear Protein Preparation and Western Blot Analysis of Acetylated Histones—Acid-soluble protein was prepared from the nuclei of cells that were harvested for cytoplasmic RNA. The nuclear pellets were thawed on ice and resuspended in 10 volumes of protein extraction buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol). Sulfuric acid was added dropwise to a final concentration of 0.4 N (0.2 M) while vortexing gently, and the nuclear lysates were incubated on ice for 1 h with vigorous vortexing every 10 min. The lysates were centrifuged at 14,000 rpm in an Eppendorf 5415C centrifuge for 10 min at 4 °C. The supernatant was precipitated with 3 volumes of 20% trichloroacetic acid on ice for 1 h with vigorous vortexing every 10 min and centrifuged at 14,000 rpm for 10 min at 4 °C. The precipitate was washed twice with acidified acetone (0.1% HCl) and twice with acetone, dried completely by desiccation for 30 min, resuspended in 200 μl of H_2O, and stored at 4 °C overnight. The concentration and purity of each acid-soluble histone protein preparation was determined using: 1) the Bio-Rad protein assay followed by measurement of A_{595} using a Shimadzu UV16OU Spectrophotometer and 2) SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining. Protein preparations were stored at −80 °C. For analysis of histone H3 and H4 acetylation levels, 15 μg of

[1] The abbreviations used are: TSA, trichostatin A; Ac-H3, acetylated histone H3; Ac-H4, acetylated histone H4; IP, immunoprecipitation; PCR, polymerase chain reaction; HDAC, histone deacetylase.

TABLE I
Primers used in chromatin immunoprecipitation experiments
For each primer set, the top primer is the upstream primer, and the bottom primer is the downstream primer.

Gene		Primer sequence
Murine *cad* promoter	mcadA	5′-TGACTAGCGGTACCGGGGTTGCTGCTGTGGAACC-3′
	3′CAD	5′-CGGGCTTGCTTACCCACCTTCCCCAGCAGTCGACAC-3′
cad coding sequence	CadF	5′-CGGGATCCGGTCAGTTCATCCTCACTCCCC-3′
	CadR	5′-CGGAATTCGGATGTACATGCCGTTCTCAGC-3′
Human *cad* promoter	hucadUS	5′-CCAGTTCCCATTGGTGTTGTTGCC-3′
	hucadDS	5′-GAGAGGCGCATCACAGAGTGGGATAA-3′
Murine *odc* gene	mODC-c	5′-CATGACGACGTGCTCGGCGTATAAGTA-3′
	mODC-d	5′-AGGTCCAGGAGCAGCTGCCTTCAG-3′
Human *tert* gene	5tel1	5-CCTTCACGTCCGGCATTCGTGG-3
	3tel1	3-AAGGTGAAGGGGCAGGACGGGT-3
Murine *albumin* gene	malbA	5′-GGACACAAGACTTCTGAAAGTCCTC-3′
	malbB	5′-TTCCTACCCCATTACAAAATCATA-3′
Murine *cdc2* gene	cdc2 358	5′-GTGGACTGTCACTTTGGTGGCTGGC-3′
	cdc2 20	5′-GGTAAAGCTCCCGGGATCCGCCAAT-3′

acid-soluble protein was used for SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis with anti-acetylated histone H3 06–599 (Upstate Biotechnology) or anti-acetylated histone H4 06–598 (Upstate Biotechnology) antibodies.

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 (20, 21). Immunoprecipitations (IPs) were performed overnight at 4 °C using 1 μg of anti-c-Myc sc-764-X (Santa Cruz), anti-acetylated histone H3 06–599 (Upstate Biotech.), anti-acetylated histone H4 06-598 or 06-866 (Upstate Biotechnology), anti-acetyl-lysine (Upstate Biotechnology), anti-phospho-H3 (Ser-10, a gift from C. David Allis), anti-N-Myc sc-791 (Santa Cruz), anti-Max sc-765-X (Santa Cruz), or no antibody, as indicated. Before the first wash, 20% of the supernatant from the IP without antibody 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 precipitation, each IP sample was resuspended in 30 μl of PCR grade TE (10 mM Tris (pH 7.5), 1 mM EDTA). Total input chromatin samples were resuspended in 30 μl of TE.

For formaldehyde cross-linking and immunoprecipitation of chromatin from mouse livers, livers were harvested from newborn and adult C57BL/6J mice. After harvesting, livers were minced with a razor blade, brought up in 20 ml of medium, and cross-linked for 10 min with 1% formaldehyde. The cross-linking reaction was stopped by addition of 0.125 M glycine. Cross-linked livers were then homogenized to individual cells using a Medi-Machine (BD Bioscience). Cells were transferred to 1.5-ml Eppendorf tubes, centrifuged at 1000 rpm in an Eppendorf 541C Centrifuge for 10 min at 4 °C to pellet the cells, which were then resuspended in cell lysis buffer, and processed for chromatin immunoprecipitation as described above.

PCR reactions contained 2 μl of IP sample or 2 μl of a 1:100 dilution of input sample, 1.2 mM $MgCl_2$, 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 of 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. Signals were quantitated using ImageQuant v4.2 (Molecular Dynamics). Fold changes in acetylation were calculated by first dividing the IP signal intensity by the input signal intensity (to control for slightly different cells numbers at each time point) for both the G_1/S sample and the G_0 sample. Then, the G_1/S value was divided by the G_0 value. The primers used for PCR analysis are shown in Table I.

RESULTS

Forced Histone Acetylation Activates the cad Promoter in Quiescent Cells—To test the hypothesis that Myc target genes are regulated via changes in histone acetylation, we first determined whether increased histone acetylation could substitute for promoter-bound Myc to activate transcription of the *cad* promoter. Quiescent NIH3T3 cells, which contain very low levels of Myc protein, were treated with the drug TSA, an inhibitor of histone deacetylases. Treatment of cells with TSA results in genome-wide increases in the levels of histone acetylation and has been shown to alter the expression of a number of genes (25). Cells were treated with increasing amounts of TSA for 16 h and harvested for isolation of histones and RNA. As shown in Fig. 2*A*, TSA treatment of NIH3T3 cells causes a large increase in the amount of acetylated histone H3 and acetylated histone H4. We then examined *cad* mRNA levels using an RNase protection assay in which a radiolabeled probe is hybridized to the *cad* message and unprotected RNA is digested with RNase A (Fig. 2*B*). We found that treatment of quiescent NIH3T3 cells with even low levels of TSA increased transcription of *cad* mRNA to approximately S phase levels. This result suggests that forced histone acetylation can bypass regulation of target genes by Myc and supports the model that recruitment of Myc may result in increased acetylation of target promoter histones.

Histone Acetylation at the cad Promoter Does Not Change as Quiescent Cells Progress through the Cell Cycle—The results obtained from treatment of NIH3T3 cells with TSA suggest that histone acetylation may play a role in activation of *cad* transcription. To test this hypothesis, we first determined whether the level of acetylation of bulk histones increased when quiescent cells re-entered the cell cycle. NIH3T3 cells were synchronized by starvation in low serum medium for 48 h and then made to progress through the cell cycle through the addition of serum. Flow cytometry was performed to ensure that cells were synchronized and progressing through the cell cycle (Fig. 3*A*). Western analysis shows that there was no increase in acetylated histone H3 or acetylated histone H4 in S phase, as compared with quiescent cells (Fig. 2*A*), although the antibodies clearly had the ability to detect acetylated histones. However, it was possible that histones bound to specific promoter regions would show a difference in acetylation. Therefore, we performed a formaldehyde cross-linking and chromatin immunoprecipitation assay to determine the levels of histone acetylation at the *cad* promoter as cells progress from G_0 to S phase. Cells were cross-linked with formaldehyde and, after sonicating the chromatin to a length of about 500 base pairs, DNA was immunoprecipitated with antibodies that recognize histones acetylated at their N-terminal tails. After immunoprecipitation, proteins were digested, and purified DNA was analyzed by PCR.

We found easily detectable levels of acetylated histone H3 on the *cad* promoter in quiescent cells when the promoter is inactive, but there was no increase in the amount of acetylated H3 bound to the *cad* promoter as cells progressed to the G_1/S phase boundary (Fig. 3*B*). However, the expected increase in binding of Myc to the *cad* promoter did occur. We also used an antibody that recognized phosphorylated H3 because it has been previously shown that histone H3 phosphorylation levels change throughout the cell cycle at immediate early gene promoters

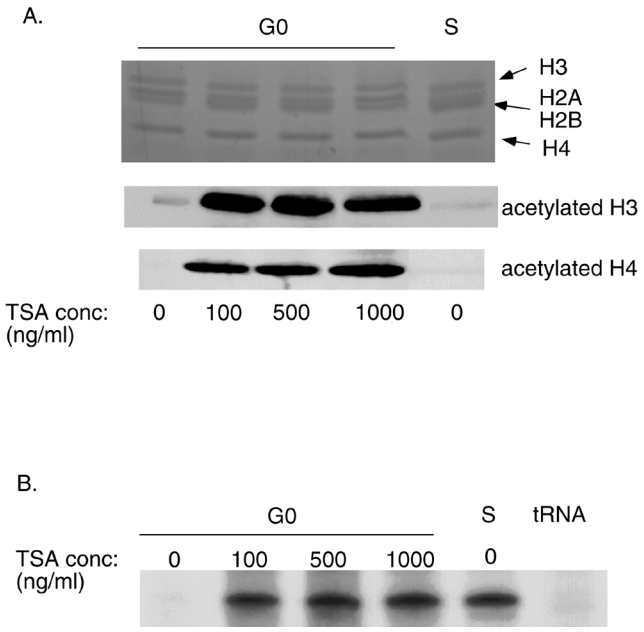


FIG. 2. **Treatment of cells with TSA stimulates *cad* transcription.** *A*, Western analysis of TSA-treated cells. Quiescent NIH3T3 cells were treated with the indicated amounts of TSA, and histones were isolated as described under "Materials and Methods." The *top panel* shows a Coomassie-stained gel showing the presence of all histones in equal amounts in each sample. The *middle* and *bottom panels* show Western blots of these samples probed with antibodies recognizing acetylated histones H3 and H4, respectively. *B*, serum-starved and stimulated NIH3T3 cells were treated with TSA as in *A*, and total RNA was isolated. *cad* mRNA levels were determined by RNase protection. To ensure even loading of RNA, RNase protections were also performed using a GADPH probe (data not shown).

(26), However, we found very low levels of histone H3 phosphorylation at the *cad* promoter in either G_0 or S phase.

To ensure that the results of the first experiment were valid, we performed a second time course and chromatin immunoprecipitation experiment. This time, we used the antibody that recognizes acetylated histone H3 and two different antibodies that recognize acetylated histone H4. As before, the amount of Myc bound to the *cad* promoter increased about 7-fold by the G_1/S phase boundary. However, we saw only modest increases in the amount of acetylated histone H3. The two different acetylated histone H4 antibodies gave slightly different results. One antibody showed no increase, whereas the other antibody showed a 3.8-fold increase in acetylation. We note that the Western blotting results shown above demonstrate the specificity of the antibodies. We have repeated the chromatin immunoprecipitation a number of times, and although we have seen varying changes in Myc binding as NIH3T3 cells progress through the cell cycle, we do not see a correlation between the increase in Myc binding and the change in histone acetylation at the *cad* promoter. The average change in acetylation at the *cad* promoter from G_0 to G_1/S is only about 1.4-fold for histone H3 and 1.4-fold for histone H4 (Fig. 3*D*). We also used a different set of primers to monitor changes in acetylation of histones H3 and H4 within the first 500 nucleotides of transcribed sequences of the *cad* gene; similar to the results shown above for the *cad* promoter, all changes observed were 2-fold or less (data not shown).

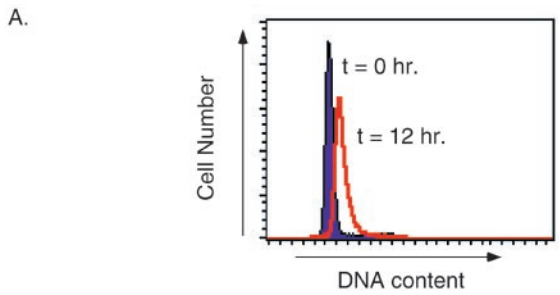
Histone Acetylation Is Not Involved in Repression of Myc Target Genes during Differentiation—Although we did see modest changes in histone acetylation on the *cad* promoter, it was unclear whether these changes were linked to transcription or whether they were simply due to cell cycle stage-specific changes in histone abundance or to general changes in chromatin structure that occur irrespective of transcription. With the goal of distinguishing between these possibilities, we turned to a second system in which Myc is known to mediate changes in gene expression. In general, differentiated cells express very low levels of Myc protein. The decrease in transcription of Myc target genes because of differentiation is a long term change, and Myc may regulate transcription during an irreversible withdrawal from the cell cycle in a different manner than it does at the G_1/S phase boundary. For example, maintaining high levels of histone acetylation on the *cad* promoter throughout the cell cycle may be necessary for the cyclical regulation of the gene. However, it is possible that histone acetylation on the *cad* promoter may be decreased when cells are terminally differentiated.

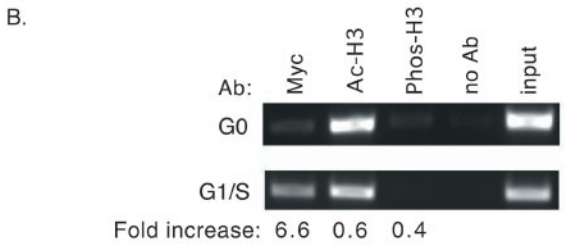
To examine histone acetylation on the *cad* promoter in a differentiation system we chose to use U937 cells, which are a human monocyte cell line. U937 cells can be made to differentiate into granulocytes with retinoic acid, and it has been previously shown that Myc mRNA and protein levels are greatly decreased after differentiation of these cells (27). U937 cells were differentiated for 5 days with 1 μM all-*trans*-retinoic acid, treated and untreated cells were cross-linked with formaldehyde, and chromatin immunoprecipitation was performed with antibodies recognizing Myc and acetylated histones H3 and H4. To ensure that cells treated with retinoic acid had exited the cell cycle, we examined the DNA content of the cells using flow cytometry and found that almost all the cells were in G_0/G_1 phase after 5 days of treatment with retinoic acid (Fig. 4*A*). Chromatin immunoprecipitation showed that, as expected, the amount of Myc bound to the *cad* promoter was greatly diminished in differentiated cells. However, the amounts of acetylated histones bound to the *cad* promoter were not different between proliferating and differentiated U937 cells (Fig. 4*B*).

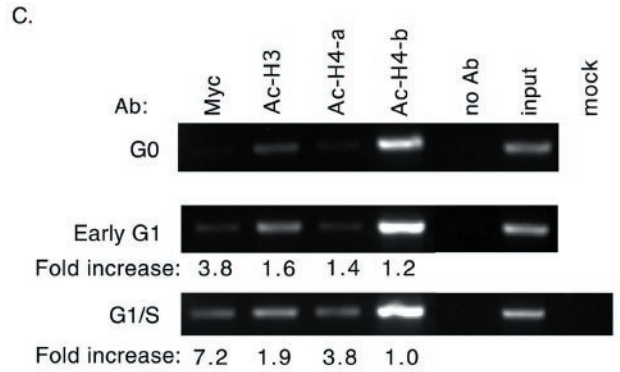
Expression of the Myc Target Gene odc Is Not Regulated by Histone Acetylation—The experiments described above establish that large changes in histone acetylation levels do not correlate with *cad* expression levels or with recruitment of Myc to the *cad* promoter. However, it remained possible that other Myc target genes could be regulated by changes in histone acetylation. Therefore, we performed PCR with primers specific to the *odc* gene using DNA precipitated by antibodies to Myc and acetylated histones. We found little change in histone acetylation at the *odc* promoter as cells progress through the cell cycle (Fig. 5). It is interesting to note that binding of Myc to the *odc* promoter is maximally increased at 4 h post stimulation, whereas Myc binding to the *cad* promoter continues to increase at 12 h after serum stimulation of quiescent cells. These data correlate well with the expression patterns of the two genes, because *odc* expression peaks much earlier in the cell cycle than does *cad* expression (28). Our results suggest that recruitment of Myc does not result in large changes in histone acetylation at either the *cad* or *odc* promoters.

Promoter Occupancy by N-Myc Does Not Lead to Changes in Histone Acetylation—The N-Myc protein has very similar DNA binding and transactivation domains as does c-Myc, and McMahon *et al.* (13) have previously shown that N-Myc can bind to TRRAP and recruit hGCN5 using the same domain as does c-Myc. Therefore, we have also tested whether recruitment of N-Myc to target promoters elicits a change in histone acetylation at those promoters. We have previously shown that the gene most responsive to increased amounts of N-Myc is the *tert* gene.[2] We have found that an approximately 20-fold increase in

[2] Mac, S. M., D'Cunha, C., and Farnham, P. J. (2000) *Mol. Carcinogen.* **29,** in press.





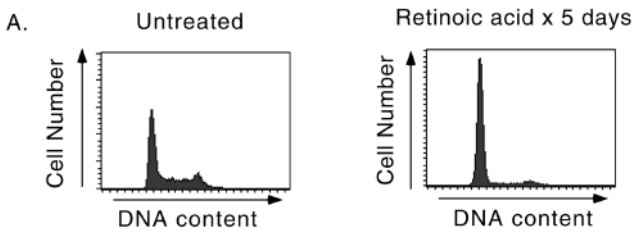


D.

	Fold Increase (G1/S to G0)		
Experiment	Myc	Acetylated H3	Acetylated H4
Expt. #1	5.8	1.5	0.5
Expt. #2	6.6	0.6	n.d.[a]
Expt. #3	7.2	1.9	3.8/1.0[b]
Expt. #4	4.0	1.5	n.d.

a) n.d.: not determined
b) Ac-H4-a/Ac-H4-b. See figure legend for definitions

FIG. 3. **Examination of acetylated histones at the *cad* promoter during the G_0 to S phase transition.** *A*, NIH3T3 cells were starved for 48 h in medium containing 0.5% serum and then stimulated with 10% serum for 12 h, which corresponds to the G_1/S phase transition. Cells were stained with propidium and analyzed by flow cytometry as described under "Materials and Methods." *B*, synchronized NIH3T3 cells were formaldehyde cross-linked, and chromatin was immunoprecipitated with antibodies against Myc, Ac-H3, or phosphorylated histone H3 (*Phos-H3*) or processed with no antibody (*no Ab*). Purified DNA was analyzed by PCR using primers recognizing the *cad* promoter (the region amplified includes from −105 to +254 relative to the transcriptional start site). *C*, repeat chromatin immunoprecipitation using antibodies to Myc, Ac-H3, and Ac-H4. Two antibodies recognizing acetylated histone H4 were used in this experiment; Ac-H4-a is anti-acetylated histone H4 06-598 (Upstate Biotechnology), whereas Ac-H4-b is anti-acetylated histone H4 06-866 (Upstate Biotechnology). Fold increases were calculated as described under "Materials and Methods." D, fold difference in histone acetylation and Myc binding seen on the *cad* promoter in multiple experiments. Fold change is defined as the ratio of G_1/S to G_0 signal seen for each antibody after normalizing to input signals, as described under "Materials and Methods." *Ab*, antibody.



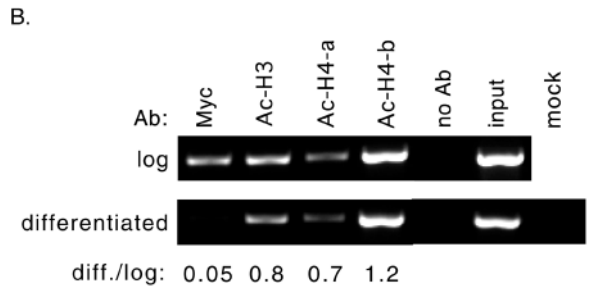


FIG. 4. **Histone acetylation at the *cad* promoter does not change after differentiation.** *A*, flow cytometry of U937 cells before and after treatment with 1 μM retinoic acid for 5 days. Cells were harvested and prepared for flow cytometry as described under "Materials and Methods." *B*, chromatin immunoprecipitation to examine histone acetylation on the *cad* promoter in proliferating and differentiated U937 cells. Cells were cross-linked with formaldehyde, and DNA was immunoprecipitated with the indicated antibodies (Ac-H4-a indicates use of 06-598 and Ac-H4-b indicates use of 06-866, both from Upstate Biotechnology). Immunoprecipitated DNA was analyzed by PCR using primers recognizing the human *cad* promoter. Ratios of IP signals from differentiated to log cells were calculated as described under "Materials and Methods." *Ab*, antibody.

binding of N-Myc to the *tert* promoter results in about a 15-fold increase in *tert* mRNA. Therefore, we have used the telomerase promoter in our experiments examining acetylation at N-Myc target genes.

Tet21N cells are neuroblastoma cells harboring a tetracycline-regulated N-Myc expression construct; addition of tetracycline to the cell culture medium shuts off expression of N-Myc. Therefore, we grew Tet 21N cells for 2 weeks in the presence or absence of tetracycline and then harvested the cells for a chromatin immunoprecipitation experiment in which antibodies to N-Myc, Max, and acetylated histones H3 and H4 were employed. As shown in Fig. 6, cells having high amounts of N-Myc have 30-fold more N-Myc bound to the *tert* promoter than do cells having low amounts of N-Myc. We have previously shown that amount of Max bound to the *tert* promoter is fairly constant, regardless of the amount of N-Myc in the cell.[2] The presence of Max at the *tert* promoter in the absence of N-Myc is likely due to promoter occupancy by Max homodimers or by Max/Mad heterodimers. As shown in Fig. 6, we again found that the amount of Max bound to the *tert* promoter changes less than 2-fold. Importantly, we found that the amount of acetylated histones bound to the *tert* promoter did not correlate with the amount of N-Myc bound. Therefore, binding of N-Myc correlates with transcriptional activity of the *tert* promoter, but the change in transcriptional activity is not mediated by a change in the amount of acetylated histones at that promoter.

Low Levels of Histone Acetylation Are Found on Nonpromoter Regions and Inactive Cell Type-specific Promoters—Our finding that the *cad*, *odc*, and *tert* promoters had high levels of acetylated histones in quiescent cells was at first surprising. To further investigate the pattern of histone acetylation on the *cad* gene, we also determined the levels of histone acetylation

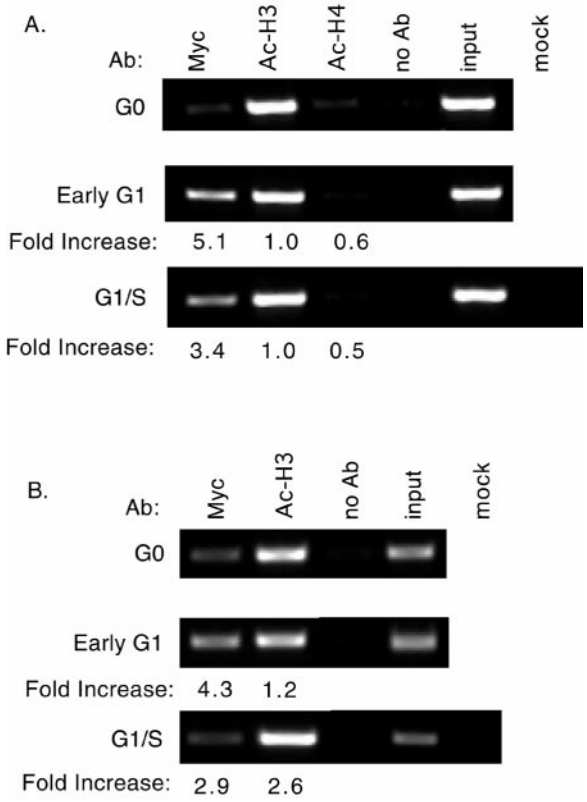


FIG. 5. **Examination of acetylated histones at the *odc* promoter during the G_0 to S phase transition.** *A*, chromatin immunoprecipitation of synchronized NIH3T3 cells using the indicated antibodies. Immunoprecipitated DNA was analyzed by PCR using primers that amplify the *odc* promoter (−52 to +357 relative to the transcription start site). Fold increase in IP PCR signals was calculated as described under "Materials and Methods." *B*, a second cell synchronization and chromatin immunoprecipitation was performed using antibodies recognizing c-Myc and acetylated histone H3. *Ab*, antibody.

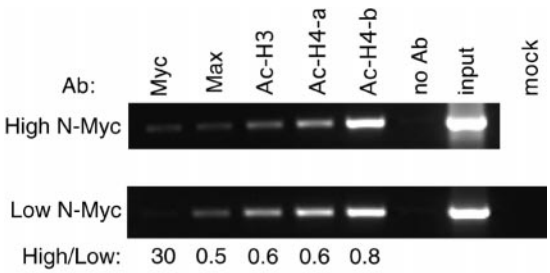


FIG. 6. **Promoter occupancy by N-Myc does not lead to changes in histone acetylation.** Tet21N cells were grown in the absence (*High N-Myc*) or presence (*Low N-Myc*) of tetracycline for 2 weeks. Chromatin immunoprecipitation was performed using antibodies to N-Myc, Max, or acetylated histones, as indicated. Immunoprecipitated DNA was analyzed by PCR using primers which amplify the *tert* promoter. Fold increase in IP signal in cells having high *versus* low amounts of N-Myc were calculated as described under "Materials and Methods." *Ab*, antibody.

on a region of the *cad* gene located approximately 6 kilobases downstream of the transcription start site but still within the transcribed sequences. As shown in Fig. 7, the acetylated histone H3 signal was greater on the *cad* promoter than on the coding region. These results, showing that high levels of acetylation are at the *cad*, *odc*, and *tert* promoters but not the *cad* coding region, suggested that high levels of histone acetylation could be a marker for promoter regions. However, when we examined acetylated histone levels at the *albumin* promoter, a cell type-specific gene that is inactive in NIH3T3 cells, we found much lower levels of histone H3 acetylation than we saw

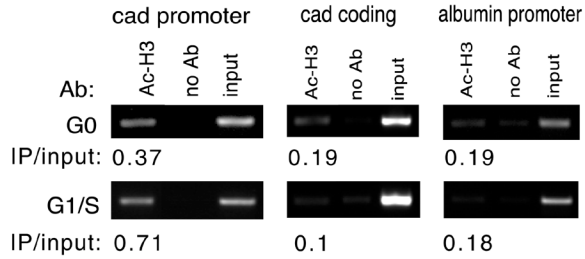


FIG. 7. **Acetylated histones are preferentially found on the *cad* promoter.** Chromatin immunoprecipitated with the acetylated histone H3 antibody from the experiment shown in Fig. 3*C* was analyzed using primers to the *cad* promoter (−105 to +254), the downstream *cad* coding region (nucleotides 6513 to 6543 of the human *cad* cDNA), and the *albumin* promoter (−540 to −189). Samples representing no antibody and 0.2% of the starting chromatin that was not immunoprecipitated (input) are also shown. *Ab*, antibody.

at the *cad* promoter (Fig. 7). In the experiment shown, the ratio of acetylated histone H3 to input chromatin was 2–7-fold higher on the *cad* promoter than on the downstream region of the *cad* gene or on the *albumin* promoter. Other experiments have consistently shown lower amounts of acetylated histones on the downstream region of *cad* and the *albumin* promoter than on the *cad* promoter (data not shown). On average, we have seen that the acetylated histone H3 signal on the *cad* promoter is approximately three times higher than the acetylated histone H3 signal on the *cad* coding region and approximately five times the acetylated histone H3 signal on the *albumin* promoter. These results suggest that high levels of histone H3 acetylation may be characteristic of a promoter that has transcriptional potential in a given cell type.

Expression of the cdc2 Promoter Is Not Regulated by Histone Acetylation—Our results with the *cad* and *odc* promoters suggested that at least some cell cycle-regulated genes maintained high levels of acetylated histones at the promoter region irrespective of the stage of the cell cycle. We wished to determine whether other cell cycle-regulated genes displayed a similar pattern of constitutive histone acetylation. We therefore examined the cell cycle-regulated *cdc2* promoter, which has been demonstrated previously to show large changes in gene expression when quiescent cells are stimulated to re-enter the cell cycle (30). We found only marginal increases in histone acetylation at the *cdc2* promoter in S phase cells, as compared with quiescent cells (Fig. 8*A*). It was unclear whether the modest changes in acetylation were linked to the transcription of the *cdc2* promoter. Therefore, we also examined levels of histone acetylation on the *cdc2* promoter in newborn and adult mouse livers. The liver of the newborn mouse is rapidly proliferating and levels of proliferation-responsive genes, such as *cdc2*, are high. However, *cdc2* expression decreases sharply as hepatocytes differentiate, dropping to almost undetectable levels by 9 weeks of age (Fig. 8*B*). We found that acetylated histone H3 levels on the *cdc2* promoter do not change significantly as *cdc2* expression decreases; in fact, the amount of acetylated histone H3 was higher in the adult liver when the promoter was inactive (Fig. 8*C*).

We note that the adult hepatocyte, although nonproliferative, does maintain proliferative potential. Removal of two-thirds of the liver by a partial hepatectomy results in the differentiated adult hepatocyte re-entering proliferative growth to restore liver mass. We have shown that *cdc2* gene expression sharply increases after partial hepatectomy (31). Thus these results again suggest that high levels of acetylated histones mark a promoter having transcriptional potential but do not imply transcriptional activity.

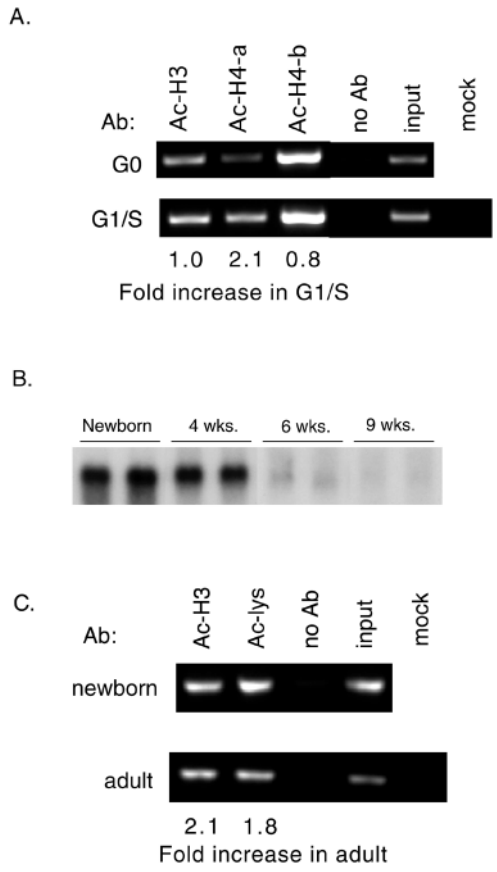


FIG. 8. **Transcription of the *cdc2* promoter does not correlate with histone acetylation.** *A*, examination of histone acetylation at the *cdc2* promoter in synchronized NIH3T3 cells. DNA from the experiment shown in Fig. 3*C* was analyzed by PCR using primers that amplify the *cdc2* promoter. *B*, Northern blot of cdc2 expression in C57BL/6J mice at various ages. The two lanes shown for each age represent duplicate loading of RNA prepared from the liver of the same animal. *C*, examination of histone acetylation on the *cdc2* promoter in newborn and adult mouse livers. Shown is a chromatin immunoprecipitation experiment on livers obtained from newborn and 20 week old mice. Chromatin from cross-linked livers was immunoprecipitated using antibodies which recognize Ac-H3 and acetylated lysine (*Ac-lys*). Fold differences in IP signals were calculated as described under "Materials and Methods." *Ab*, antibody.

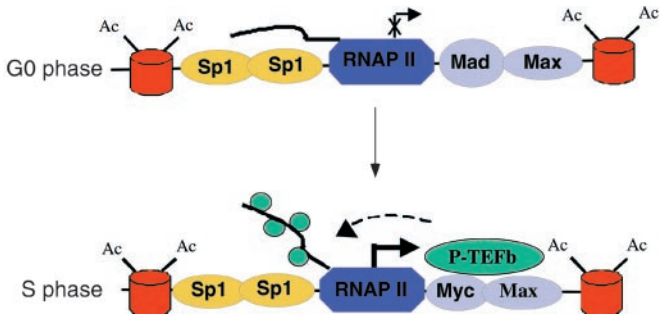


FIG. 9. **Alternative model for Myc-mediated transcriptional activation.** In G_0 phase, Mad/Max, Sp1, and RNA polymerase II are bound to the *cad* promoter and the nucleosomes have high levels of acetylated histones. In S phase, Myc/Max is bound to the *cad* promoter and recruits P-TEFb, which phosphorylates the RNA polymerase II CTD and allows elongation of *cad* transcripts.

DISCUSSION

Previous studies have suggested that an exchange in promoter occupancy of Mad/Max heterodimers for Myc/Max heterodimers can lead to activation of Myc target genes. However, the mechanisms by which promoter-bound Myc can activate transcription are not yet fully understood. A recently identified Myc-interacting protein called TRRAP has been shown to interact with a histone acetyltransferase (13). Others have shown that changes in the amount of acetylated histones correlate with transcriptional activation of certain promoters. For example, virus infection and hormonal stimulation can cause increases in promoter activity and increases in histone acetylation (32, 33). Studies such as these have led to the hypothesis that Myc recruits TRRAP, which in turn recruits histone acetyltransferases, and this leads to increased histone acetylation at the promoter and increased transcriptional activity. Although an attractive model, there is no direct evidence showing that promoter-bound Myc can influence the amount of acetylated histones.

Using a chromatin immunoprecipitation assay, we have performed experiments designed to test whether recruitment of Myc family members to a promoter results in increased histone acetylation. We have shown large changes in the amount of c-Myc bound to the *cad* promoter when quiescent cells re-enter the cell cycle and when proliferating cells exit the cell cycle. The histone acetylation model predicts that the *cad* promoter would have low levels of acetylated histones in quiescent and differentiated cells but high levels in proliferating cells. However, we find only modest changes in the amount of acetylated histones on the *cad* promoter or on the DNA located within 500 base pairs downstream of the transcription start site, during these cell cycle transitions. Similarly, we do not see large changes in the amount of acetylation on the *odc* promoter, another Myc target gene, when quiescent cells re-enter the cell cycle. Finally, large changes in promoter occupancy by N-Myc do not lead to changes in the levels of acetylated histones. Our results suggest that changes in acetylation are not correlated with transcriptional activity of Myc target genes. Others have reported similar results for different cellular genes. For example, O'Neill and Turner (34) have shown that there is no correlation between the amount of acetylated histone H4 bound to specific cellular genes and the transcriptional activity of those gene in proliferating *versus* differentiated mammalian cells. Thus, it appears that acetylation may be a critical determinant of transcription rate only in specific regulatory pathways.

Our studies have shown 2-fold increases in the amount of acetylated histones at the *cdc2* promoter when the transcriptional activity both increases (*i.e.* from the G_0 to S phase transition) and decreases (*i.e.* upon differentiation of hepatocytes). Thus, transcriptional activity of the *cdc2* promoter also appears to be regulated independently from changes in histone acetylation. *Cdc2* is regulated by members of the E2F family of transcription factors, and a recent study found 2–6-fold changes in the amount of acetylated histones on several E2F-regulated target genes in S phase as compared with quiescent cells (35). However, that study did not demonstrate that the changes in acetylation were necessary for transcriptional activation. In a previous study, Krebs *et al.* (36) found that cell cycle-regulated changes in acetylation did occur at the yeast HO promoter but that these changes were independent of transcription from that promoter. Perhaps the modest increase in histone acetylation that we observed at the *cad* and *cdc2* promoters in S phase could be related to general cell cycle changes rather than to specific transcriptional activity.

Several questions arise from our studies. First, if acetylation is not regulating the proliferation-related changes in *cad* gene expression, why did *cad* mRNA increase after the treatment of quiescent cells with TSA? The *cad* promoter contains two Sp1 binding sites, located just upstream of the start site of transcription, which are critical for determining the location of the transcription start site and for basal promoter activity. Sp1 can interact with both histone deacetylases and histone acetylases

(37, 38). Perhaps, under normal conditions, Sp1/HDAC and Sp1/histone acetyltransferase complexes compete for binding to target promoters, allowing an intermediate amount of acetylation. The binding of Sp1 to target promoters has been shown to be constitutive through the cell cycle, suggesting that the amount of acetylated histones may not normally change on an Sp1 target gene in different stages of the cell cycle. However, the addition of TSA, a histone deacetylase inhibitor, can shift the balance between deacetylase and acetylase activities and cause artificially elevated levels of acetylation at Sp1 target genes (39, 40). Of course, it is also possible that the effect of TSA on *cad* mRNA levels was due to an indirect effect.

A second question that arises from our studies is whether Myc does actually recruit acetylases to target promoters but that their function is to acetylate something besides histones. For example, general transcription factors (41), site-specific transcription factors (42–44), and HMG proteins (45) can all be acetylated. We have tried to address this possibility by performing two additional experiments. First, we repeated the chromatin immunoprecipitation results using an antibody that recognizes all acetylated lysines, not just acetylated histones. Although we did find a modest increase in acetylation at the *cad* promoter using this antibody (data not shown), the conclusions were complicated by the already high levels of acetylated histones on the promoter. It remains possible that the degree of acetylation of a nonhistone protein changes when Myc binds, but proof awaits the development of acetylation state-specific antibodies to candidate proteins. We next reasoned that if the role of Myc was to increase acetylation of some component of the transcription complex then targeted recruitment of a histone acetyltransferase to the *cad* promoter should cause transcriptional activation. We have recently shown that Gal4-CBP can cause transcriptional activation of certain cellular promoters (46). However, recruitment of Gal4-CBP was not able to induce transcription of the *cad* promoter (data not shown). Thus, neither experiment provided evidence in support of Myc-mediated changes in acetylation.

Finally, if Myc is not functioning via changes in acetylation, then what might be the mechanism by which recruitment of Myc leads to transcriptional activation? It has been proposed that regulation of many genes can occur at the level of promoter escape (47). In other words, site-specific transcription factors and RNA polymerase may be bound to the inactive promoter, and the recruitment of Myc would stimulate the transition from initiation complex formation to elongation (Fig. 9). Preliminary chromatin immunoprecipitation experiments using an antibody to RNA polymerase II have shown equivalent levels of RNA polymerase bound to the *cad* promoter in quiescent or S phase cells.[3] Thus, it is possible that Myc regulates transcription by stimulating a prebound transcription complex to begin elongation. Others have suggested that activator-dependent recruitment of a kinase called P-TEFb to the complex results in phosphorylation of the CTD and transition to elongation (29). P-TEF-b is composed of cdk9 and cyclins T1, T2a, T2b, or K. Future studies investigating whether recruitment of any of these subunits correlate with binding of Myc are in progress.

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[3] S. R. Eberhardy, unpublished data.

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