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Recruitment of Gcn5-containing Complexes during c-Myc-dependent Gene Activation

STRUCTURE AND FUNCTION ASPECTS*

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From the ‡Section for Natural Sciences, Södertörns Högskola, Box 4101, Huddinge 141 04, Sweden, the §Department of Biosciences, Karolinska Institutet, Huddinge 141 57, Sweden, the ‖Department of Biochemistry and Molecular Genetics, University of Virginia School of Medicine, Charlottesville, Virginia 22908, and the ‡‡Howard Hughes Medical Institute and Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, Pennsylvania 16802

The N-terminal domain of c-Myc plays a key role in cellular transformation and is involved in both activation and repression of target genes as well as in modulated proteolysis of c-Myc via the proteasome. Given this functional complexity, it has been difficult to clarify the structures within the N terminus that contribute to these different processes as well as the mechanisms by which they function. We have used a simplified yeast model system to identify the primary determinants within the N terminus for (i) chromatin remodeling of a promoter, (ii) gene activation from a chromatin template *in vivo*, and (iii) interaction with highly purified Gcn5 complexes as well as other chromatin-remodeling complexes *in vitro*. The results identify two regions that contain autonomous chromatin opening and gene activation activity, but both regions are required for efficient interaction with chromatin-remodeling complexes *in vitro*. The conserved Myc boxes do not play a direct role in gene activation, and Myc box II is not generally required for *in vitro* interactions with remodeling complexes. The yeast SAGA complex, which is orthologous to the human GCN5-TRRAP complex that interacts with Myc in human cells, plays a role in Myc-mediated chromatin opening at the promoter but may also be involved in later steps of gene activation.

The c-Myc protein (Fig. 1) is a regulator of several important cellular processes. These include the control of normal cell growth, differentiation, and apoptosis. Elevated levels of c-Myc have been shown to play a role in tumorigenesis, probably through the altered control of genes required for cellular proliferation (1, 2). c-Myc is a member of a family of transcription factors characterized by leucine zipper and basic helix-loop-helix dimerization and DNA binding domains (3–6). c-Myc heterodimerizes with Max, another leucine zipper-basic helix-loop-helix protein. The Myc-Max heterodimer is a high affinity DNA binding complex, which binds to E boxes within promoters/enhancers and is a potent activator of gene expression and cellular transformation. Many studies have identified genes that are activated by c-Myc. These include genes encoding proteins required for cell cycle, growth, and apoptosis as well as proteins involved in stress response, amino acid transport, and cell adhesion (reviewed in Ref. 7). c-Myc can also repress target genes, and indeed c-MycS, which lacks most of the activation domain, is still able to repress genes and participate in cellular transformation (8).

Kato *et al.* (9) showed that the N-terminal 143 amino acids of c-Myc constitute the activation domain and play an important role in cellular transformation. However, the c-Myc activation domain appears to be a complex structure, since it mediates several other functions in addition to gene activation. Li *et al.* (10) showed that sequences within the N-terminal 143 amino acids are also involved in the transcriptional repression activity of c-Myc. Furthermore, the conserved Myc boxes within the activation domain have been implicated in targeted proteolysis of the Myc protein via the proteasome (11–14). The Myc boxes also play a key role in modulating c-Myc activity in response to cellular signaling systems. Consistent with this functional complexity, the N-terminal domain of c-Myc has been reported to interact with several other proteins, such as TATA-binding protein (15), p107 (16, 17), α-tubulin (18), Pam (19), MM-1 (20), Bin1 (21), AMY-1 (22), TRRAP (23), and Gcn5 (24). Binding to TATA-binding protein has been shown to be important for gene activation by c-Myc *in vitro* (25).

Interestingly, the TRRAP and Gcn5 proteins have been identified in mammalian protein complexes that display histone acetyltransferase (HAT)[1] activity (26–28). Deletion of Myc box II causes loss of both interaction with TRRAP/Gcn5 and of cellular transformation potential (23, 24). These observations have led to an attractive model in which Gcn5 complexes are recruited to target promoters and help to remodel the promoter chromatin structure into an active form. Others have reported that Mad-Max heterodimers, which also bind to E boxes, recruit histone deacetylase complexes, leading to deacetylation of

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[1] The abbreviations used are: HAT, histone acetyltransferase; GST, glutathione *S*-transferase.

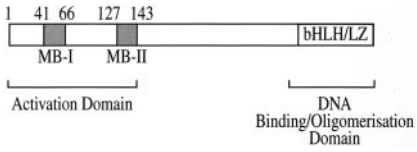


FIG. 1. **Diagram locating the activation domain within the c-Myc protein.** The evolutionarily conserved Myc boxes I and II (*MB-I* and *MB-II*) are *shaded*. MB-I is an important hot spot for cancer-related mutations.

promoters and gene repression (29). Thus, depending on the balance of E box occupancy between Myc-Max and Mad-Max, target genes will either be activated or repressed. The key role of Myc box II in this model has been extended by the observation that this region also plays a critical role in promoter recruitment of ATP-dependent chromatin-remodeling complexes by Myc (30). It has been shown that ATP-dependent complexes and histone acetyltransferases collaborate during gene activation (31).

Although this model remains attractive, there are two main problems with regard to the role of c-Myc. First, Myc box II, which has been suggested to be a key determinant for the interaction with Gcn5-containing and ATP-dependent remodeling complexes and thus to play a key role in subsequent gene activation, has previously been associated with the gene repression activity of c-Myc (8, 10). Second, recent studies have shown that the acetylation status of some Myc-regulated promoters does not change in response to their activation by mitogen treatment (32). Therefore, since recruitment of histone acetyltransferase complexes is considered to be an important component of Myc-mediated gene activation, it is necessary to consider whether processes, other than promoter acetylation, might be modulated by recruited Gcn5 complexes.

Gcn5-containing complexes have been extensively purified from yeast, where their role in gene activation has been studied both *in vitro* and *in vivo*. Comparison with the mammalian complexes shows that the yeast SAGA complex is highly homologous to the human complexes that have been described (33). Human c-Myc is an efficient activator of gene expression in yeast, and the N-terminal activation domain is active in yeast when fused to a heterologous DNA binding domain (34). Thus, biochemical and genetic approaches available for yeast offer the opportunity to study interaction of c-Myc with highly purified Gcn5-containing complexes *in vitro* as well as the functional consequences of the interaction *in vivo* on chromatin templates in a relatively simple and well defined system. In this report, we define primary determinants within the c-Myc activation domain that are involved in chromatin remodeling, overall gene activation, and interaction with Gcn5 complexes as well as other chromatin-remodeling complexes. Further, we suggest that Gcn5 complexes and ATP-dependent remodeling complexes may effect transcriptional processes that lie downstream of the promoter chromatin remodeling step.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media

YS33 (*MAT***a** *his3–11,15 leu2–3,112 ura3-Δ5 Δpho80*::*HIS3 Δpho4*::*ura3-Δ5* Can^R (35) was used for chromatin opening analysis. Pho80 is a high phosphate-dependent repressor of Pho4; by deleting it, the requirement for low phosphate conditions for Pho4 activity is avoided. YS5339 is a derivative of YS33, with the *GCN5* gene deleted. The Ada-SAGA complex components (*ada1*, *ada2*, and *gcn5*) were deleted from the strain PSY316 (*MATα ade2–101 ura3–52 leu2–3,112 his3-Δ200 lys2*) by insertion of the *hisG* gene. The Ada-specific component Ahc1 (36) was disrupted with Kan-MX from the strain BY4741 (*MAT***a** *his3-Δ1 leu2-Δo met15-Δo ura3-Δo* (Research Genetics)). FY60 (*MATα his4–9178 ura3–52 leu2Δ1*), FY295 (*MATα his4–9178 ura3–52 leu2Δ1 lys2–173R2 spt3–202*), and FY1291 (*MAT***a** *ura3–52 leu2Δ1 lys2–173R2 trp1Δ63 arg4–12 spt20Δ200::ARG4*) were used to investigate SAGA-specific activity and were provided by F. Winston (Harvard Medical School). The *snf6*-deleted strain (CY332) was produced from CY26 (*MATα his3-Δ200 ura3–52 leu2Δ1 trp1-Δ1 lys2–801 ade2–101*) both were provided by C. Peterson (University of Massachusetts Medical School). The *pho4*, *snf2*-deleted strain (CY408) was produced from CY338 (*MAT***a** *ura3–52 lys2–801 ade2–101 leu2-Δ1 his3-Δ200 pho4*::*URA3*), both kindly provided by W. Hörz. Investigation of chromatin opening in the absence of SAGA and/or SWI/SNF complexes was performed in FY1551 (*MAT***a** *ura3–52 leu2Δ1 his3 Δ 200 ada2 Δ*::*HIS3 snf2 Δ*::*LEU2*) and a congenic WT (FY3) kindly provided by F. Winston (Harvard Medical School).

Yeast transformations were performed using the method of Ito *et al.* (37). Selective yeast medium was SD-LU (0.67% (w/v) yeast nitrogen base without amino acids (Bio 101), 2% (w/v) glucose (Sigma), 0.2% (w/v) drop out mix without uracil and leucine (38). In the cases where yeast strain contained the *PHO80* gene, transformants were grown on a phosphate-free medium (39) prior to activity or opening assays. However, our results have shown that this was an unnecessary precaution as the Myc-Pho4 fusion proteins are not affected by the Pho80 negative regulator in high phosphate conditions, and we obtained the same results after growth in SD medium.

Plasmids

c-Myc 1–149 was expressed from plasmids pP472S and pP472L. Plasmid pP472S (2μ *URA3* Amp^R) contains the 1.1-kb *Ava*I *PHO4* fragment (Pho4DBD) and the *PHO4* promoter and is a mutated form of YEpΔ2 (40). The original *Sac*I site was deleted, and a new *Sac*I site was added upstream of sequence encoding the Pho4DBD and downstream of the *PHO4* promoter. The c-Myc N-terminal sequence (residues 1–149) was amplified by PCR from a full-length clone and has *Sac*I ends. Plasmid pP472L (2μ *LEU2* Amp^R) is the same as pP472S, but the *Sac*I site has not been moved. The 1–149 insert can be moved between the two plasmids using the unique *Hin*dIII and *Bam*HI sites. The reporter plasmid for functional assays of the 1–149P fusion protein and its derivatives was pPZleu (*ARS CEN LEU2* Amp^R) with a 2.2-kb *Bam*HI-*Dra*I *PHO5* promoter fragment cloned upstream of the *lacZ* gene. Nucleosomes assemble in the correct pattern on the *PHO5* promoter (41). The reporter plasmid for ΔTATA chromatin opening assays was pΔTATAleu (*ARS CEN LEU2* Amp^R). Expression of full-length *myc* and *max* genes was controlled by the GAL1/10 promoter in plasmids pSD-Myc (ARS, CEN, TRP1, AmpR) (42) and pRSmax9 (ARS, CEN, LEU2, AmpR) (43), respectively. The reporter plasmid for measuring intact c-Myc activity assays was pPHO5UASLSCYC (2μ URA3 AmpR) (43). Deletions made in the context of the 1–149P fusion protein and the intact c-Myc protein have been described previously (12). Construction of pGSTTMyc143 is described elsewhere (44).

Preparation of Yeast Extracts and β-Galactosidase Assay

Yeast cells carrying the expression and reporter plasmids were grown in 30 ml of selective glucose medium to a density of 1×10^7 cells/ml. Cells were harvested, and extracts were prepared according to Green *et al.* (45) in 150 μl of buffer Z (120 mM $NaPO_4$, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Small scale total protein (Bio-Rad) and β-galactosidase (46) assays were performed in microtiter plates and measured with a microplate reader (Bio-Rad or Molecular Devices).

Analysis of Chromatin Remodeling at the PHO5 Promoter

Isolation of Nuclei—Nuclei were isolated from yeast cultures following the procedure described by Svaren *et al.* (35). Briefly, a 500-ml culture was grown to midlog phase ($2–4 \times 10^7$ cells/ml). The cells were washed and spheroplasted using 700 units of yeast lytic enzyme (ICN) at 30 °C. The spheroplasts were lysed in Ficoll solution (18% (w/v) Ficoll, 20 mM KH_2PO_4, pH 6.8, 1 mM $MgCl_2$, 0.25 mM EGTA, 0.25 mM EDTA), and the nuclei were pelleted by centrifugation.

ClaI Accessibility Assay—Nuclei containing about 10 μg of DNA were digested for 30 min at 37 °C with 160 or 400 units of *Cla*I. To monitor cleavage at the *Cla*I site, DNA was isolated and digested with 50 units of *Hae*III. The DNA was resolved on a 1.5% agarose gel and blotted onto Hybond N membrane (Amersham Pharmacia Biotech). The probe used for hybridization was probe D (47), labeled with [α-^{32}P]dCTP using random hexanucleotides and the Klenow fragment of DNA polymerase (Amersham Biosciences). This probe recognizes the 1.38-kb *Hae*III or 1.07-kb *Hae*III/*Cla*I fragments of the *PHO5* promoter. To monitor remodeling of the *PHO5* promoter in a plasmid context, an upstream *Hin*dIII-*Bam*HI upstream fragment from pPZleu was used as a probe. DNA isolated after cleavage with *Cla*I was cleaved with *Eco*RV instead

of *Hae*III in these assays. The intensity of hybridization to the *PHO5* DNA fragments was measured using a phosphor imager (Fuji FLA-3000).

Purification of HAT and SWI/SNF Complexes

Yeast whole cell extracts and isolation of HAT complexes were performed as described by Grant *et al.* (48). Further purification of the SAGA, Ada, NuA4, and NuA3 complexes was as described by Grant *et al.* (49), except that the order of columns was modified. Each complex was purified over Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen), followed by MonoQ HR 5/5 (Amersham Biosciences), MonoS HR 5/5 (Amersham Biosciences), histone agarose (Sigma), and Superose 6 HR 10/30 (Amersham Biosciences) columns. The SWI/SNF complex, containing HA-tagged Snf2, was isolated from yeast extracts as described by Côté *et al.* (50).

GST Pull-down and HAT Assays

Each HAT complex was incubated in PDB (150 mM NaCl, 50 mM HEPES, pH 7.5, 10% glycerol, 0.1% Tween 20, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) with the indicated GST fusion protein for 2 h at 4 °C while rotating on a wheel. The supernatant was removed, and beads were washed four times in PDB. For the HAT complex assays, equal fractions of both supernatants and beads were directly assayed for nucleosomal acetyltransferase activity as described previously (49). The SWI/SNF complexes were detected by immunoblotting using anti-HA antibodies (51). Expression and purification of the GST-Myc143 fusion protein has been described previously (44).

RESULTS

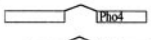
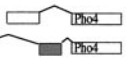
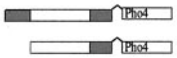
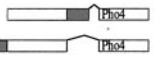
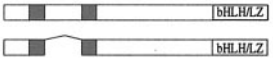
Mapping of Activation Domain Regions Involved in c-Myc-dependent Gene Activation—To determine which regions of the c-Myc activation domain are involved in gene activation, a number of derivatives of the activation domain were fused to the oligomerization/DNA-binding domain of the yeast Pho4 protein as described in Ref. 12. The activity of the different fusion proteins was then measured by their ability to activate a *lacZ* reporter gene, which was fused to the Pho4-responsive *PHO5* promoter. The experiments were performed in a yeast strain that lacks the *PHO4* gene, and thus lacks the normal Pho4 protein. Fig. 2*A* shows that gene activation potential lies within the regions outside the conserved Myc boxes, namely residues 1–41 and 66–127. Both of these fragments have an autonomous activation potential, whereas, in contrast, the construct containing only the Myc boxes has no measurable activity. Furthermore, the reduced activity of the 94–127 fragment shows that full activity of 66–127 requires sequences spanning residue 94. All of the constructs needed to draw these conclusions are expressed at similarly high levels in yeast, while the remaining constructs, containing one or both Myc boxes, are expressed at much lower levels due to the role of the Myc boxes in targeting proteasome-mediated degradation (12). The results from these latter constructs further support the importance of the 1–41 and 66–127 fragments in gene activation, since there is little or no change in activation potential when either of the Myc boxes are deleted compared with constructs containing both.

The predominant activation potential lies in the 66–127 fragment. The importance of this fragment could be confirmed in the context of the intact c-Myc protein, which can bind to the Pho4 binding sites in the *PHO5* upstream activating sequence in the context of a *CYC1-lacZ* reporter gene as a heterodimer with the Max protein (42, 52). As shown in Fig. 2*A*, the construct lacking this region has a 6-fold lower activation potential compared with the intact c-Myc protein. In contrast, deletion of one or both Myc boxes does not significantly reduce activation potential, either in the context of intact c-Myc or the derivative lacking residues 66–127.

We chose to use a reporter gene based on the *PHO5* promoter because chromatin remodeling within this promoter during gene activation has been extensively studied (53). This is appropriate in the light of previous reports showing recruitment of chromatin remodeling activities by the c-Myc activation domain (23, 24, 30). The status of the *PHO5* promoter can be monitored quantitatively by isolating chromatin and measuring the proportion of promoters that can be cleaved by *Cla*I. The *Cla*I site is occluded by a nucleosome in the repressed promoter but becomes available for cleavage in the remodeled promoter. Fig. 2*B* shows that the intact c-Myc activation domain is able to mediate opening of the *PHO5* promoter and further that this occurs equally well using a nonexpressed *PHO5* reporter gene from which the TATA box has been deleted. Thus, the observed remodeling is not an indirect consequence of reporter gene transcription. This assay was used to measure the chromatin remodeling potential of the different c-Myc fusion proteins described above. The results, shown in Fig. 2*A*, generally reflect the potential of the same constructs to activate the reporter gene. They further support the predominant role of residues 1–41 and 66–127 as well as the view that the Myc boxes do not appear to play a direct role in chromatin remodeling, at least at this promoter.

Interaction of the c-Myc Activation Domain with Chromatin-remodeling Complexes—It has been reported that the c-Myc activation domain interacts with Gcn5-containing histone acetyltransferase complexes in order to recruit them to Myc-activated target genes (23, 24). Since Gcn5 complexes are highly conserved between yeast and mammalian cells, we tested the activation potential of Pho4 fusion proteins containing the intact activation domain (1–149) as well as its isolated 1–41 and 66–127 deletion derivatives in *ada2*, *ada3*, and *gcn5* mutant yeast strains that lack functional Gcn5 complexes (49). The activity of all constructs was severely reduced in the mutant strains, indicating that both the 1–41 and the 66–127 activation domain modules require Gcn5 complexes for full activity, irrespective of the presence or absence of the Myc boxes (Fig. 3*A*). In light of previous reports showing that Myc box II is required for interaction with Gcn5 containing complexes in mammalian cell-free extracts, we decided to investigate the interaction of purified Myc activation domain derivatives with purified Gcn5-containing complexes *in vitro*. In yeast, two main Gcn5-containing complexes have been identified, the SAGA complex and the Ada complex. c-Myc might be expected to interact with the SAGA complex, since this complex contains the Tra1 protein, the yeast homologue of the mammalian TRRAP protein that has been implicated in Gcn5 recruitment by Myc in mammalian cells (23). Fig. 3*B* shows that both complexes interact with the intact c-Myc activation domain *in vitro*. However, under the same conditions, interaction of the 66–127 fragment with the SAGA complex is greatly reduced, and interaction with the Ada complex is not detectable. This is consistent with the recent observation that a larger c-Myc fragment is also required for efficient interaction with the TATA-binding protein (44). Interestingly, Myc box II (residues 128–143) is required for interaction with the Ada complex but not the Tra1-containing SAGA complex. To permit interpretation of the specificity and significance of these results in a broader context, we also measured interactions with some other purified chromatin-remodeling complexes. The NuA3 complex has not previously been shown to interact with activator proteins, and it did not interact with the c-Myc activation domain in our experiments. The NuA4 complex is a histone H4 HAT, which also contains the Tra1 protein and can be recruited to promoters by several activators during gene activation (54, 55). NuA4 interacts with the c-Myc activation domain in a way similar to the SAGA complex. (Note that the NuA4 fraction used is contaminated with a histone H3 HAT activity but that NuA4 interaction can be unambiguously measured by its his-

A

Name	Construct	Relative β-galactosidase activity (± SD)		Relative chromatin remodelling activity (±SD)	
Pho4 fusion proteins					
1-41P		**60**	(7.3)	**150**	(4.2)
66-127P		**140**	(9.7)	**148**	(13)
94-127P		**13**	(3.6)	**58**	(1.5)
MBI+IIP		**2.1**	(0.8)	**8**	(0.5)
Pho4DB		**2**	(0.5)	**15**	(0.5)
1-149P		**100**		**100**	(7.0)
ΔMBIP		**93**	(1.7)	**107**	(0.5)
ΔMBIIP		**100**	(5.0)	**92**	(16)
41-149P		**39**	(4.5)	**104**	(2.4)
66-149P		**38**	(3.7)	**81**	(7.5)
41-127P		**31**	(5.1)	**92**	(16)
Full-length myc					
full-length		**100**			
Δ66-127		**17**	(6.0)		
ΔMBI		**90**	(23)		
ΔMBI+II		**149**	(28)		
Δ41-149		**18**	(2.6)		

B

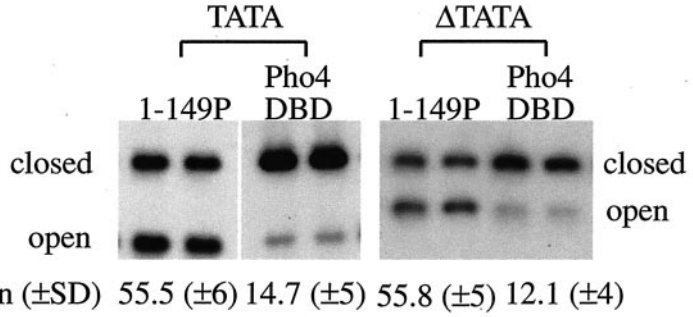


FIG. 2. **Identification of determinants for gene activation and chromatin remodeling within the c-Myc activation domain.** *A*, the gene activation and chromatin opening potency of deletion derivatives of the c-Myc activation domain are shown ± S.D. The values are shown relative to the activity of the 1–149P construct (*upper panel*) or the full-length construct (*lower panel*). The activation domain derivatives are fused to the DNA binding and oligomerization domains of the Pho4 protein (*upper panel*) or are assayed in the context of the full-length protein in cells expressing the Max protein (*lower panel*). The residues included in each construct are evident from the construct name, and each construct is also shown in *diagram form*. The *shaded regions* represent the conserved Myc boxes I and II (residues 42–65 and 128–149, respectively). *B*, the c-Myc activation domain is needed for efficient chromatin remodeling of the *PHO5* promoter, and remodeling is not dependent on transcription of the *PHO5* gene. The *diagram* shows the repressed *PHO5* promoter. The four positioned nucleosomes covering the promoter that are subject to remodeling are shown (*gray circles*). The *small black and white circles* represent binding sites for the Pho4 protein and the c-Myc/Max heterodimer. The *arrow* indicates a *Cla*I recognition site that is occluded in the repressed promoter but that is available for cleavage in chromatin from derepressed cells. The amount of cleavage by 120 and 400 units of *Cla*I shows the proportion of promoters that have been remodeled (open) in relation to those that have not been (closed). The amount of remodeling is shown for each construct ± S.D. ($n = 4–6$). The ΔTATA strain contains a *PHO5* promoter from which the TATA box has been deleted, leading to a lack of measurable transcription from the promoter under derepressed conditions.

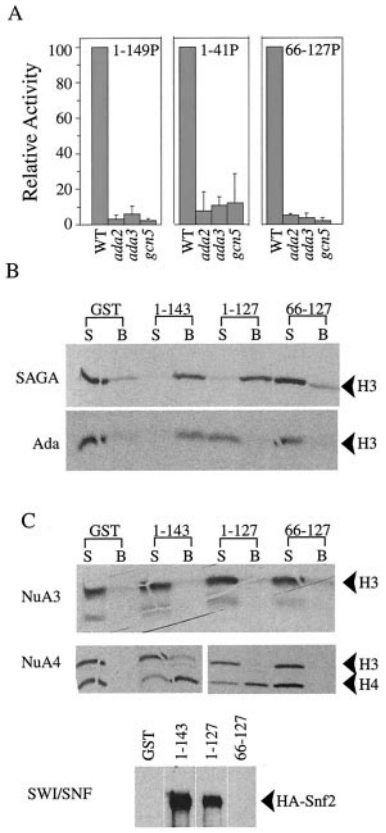


FIG. 3. **Identification of determinants required for direct interaction between the c-Myc activation domain and chromatin-remodeling complexes.** *A*, histograms comparing the affect of *ada2*, *ada3*, and *gcn5* mutations on the gene activation activity of the intact activation domain (residues 1–149) and its constituent modules (residues 1–41 and 66–127). The activity in the mutant strains is expressed as a proportion of the activity of the respective Myc derivatives in the corresponding wild type strain. *B*, fluorograms showing the interaction of purified GST, alone or fused to the indicated regions of the c-Myc activation domain, with purified Gcn5-containing complexes (SAGA and Ada). The distribution of the complexes in the bound (*B*) and unbound (*S*) fractions was assayed by their ability to acetylate histones within mononucleosomes substrates with [^{3}H]acetyl-CoA. The *arrows* indicate bands representing acetylated histones, histone H3 (*H3*) in this case. *C*, incubation of GST proteins under the same conditions as in *B* with purified NuA3, NuA4, and SWI/SNF complexes. Note that the NuA4 preparation is contaminated with a histone H3-acetylating complex; NuA4 is specific for histone H4. Annotation of the *upper* and *middle panels* is as for *B*. In the *lower panel*, only the bound fractions are shown. Association of the SWI/SNF complex with the respective GST proteins was detected using Western blots and developed with an antibody against the HA epitope tag. The purified SWI/SNF complex used contained an HA tag fused to the Snf2 subunit.

tone H4 acetyltransferase activity.) As for SAGA, the interaction does not require Myc box II. The same pattern was also seen for interaction with the SWI/SNF complex, a member of the ATP-dependent class of chromatin-remodeling complexes. These studies show that efficient interaction with a range of chromatin-remodeling complexes requires a fragment larger than the 66–127 fragment. However, Myc box II is not generally required for direct interactions of the c-Myc activation domain with recruited protein complexes, although it does play an important role in the interaction with the Ada complex.

The SAGA and SWI/SNF Complexes Are Important for c-Myc Function in Vivo—The *ada2*, *ada3*, and *gcn5* mutant strains used to show the importance of Gcn5-containing complexes *in vivo* in earlier experiments disrupt both the SAGA and the Ada complex. To determine which Gcn5 complex is important for the activation potential of c-Myc *in vivo*, we used mutants that cause specific defects in the individual complexes.

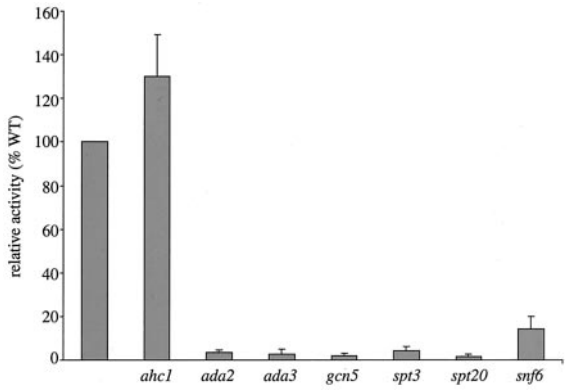


FIG. 4. **The SAGA Gcn5-containing complex is specifically important for Myc-mediated gene activation *in vivo*.** Shown is the ability of the 1–149P Myc-Pho4 fusion protein to activate a *PHO5-lacZ* reporter gene in wild type and mutant yeast strains. The *ada2*, *ada3*, and *gcn5* mutations cause defects in both the SAGA and Ada complexes. Defects resulting from the *spt3* and *spt20* mutants are specific for the SAGA complex. The *ahc1* and *snf6* mutations each cause specific defects in the Ada and SWI/SNF complexes, respectively.

Fig. 4 shows that mutations affecting the Spt3 and Spt20 proteins that are specific for the SAGA complex cause similar reductions in activity to the *ada2*, *ada3*, and *gcn5* mutations that affect both complexes. Furthermore, deletion of the *AHC1* gene, which causes defects that are specific for the Ada complex, has little effect on the activation potential of the c-Myc activation domain. If anything, activation is increased in this strain, consistent with recent studies of another activator in an *ahc1* mutant strain (56). We thus conclude that the SAGA complex is the Gcn5-containing complex that is responsible for Myc-dependent gene activation, at least for this promoter. ATP-dependent chromatin-remodeling complexes have also been implicated in Myc-dependent gene activation (30). Since we observed a direct interaction of the c-Myc activation domain with the SWI/SNF complex (Fig. 3*C*), we used a yeast mutant strain defective in the Snf6 subunit of the SWI/SNF complex to determine whether ATP-dependent complexes might be important in our model system. Fig. 4 shows that the Snf6 protein is important for efficient gene activation by the c-Myc activation domain.

Requirement for the SAGA and SWI/SNF Complexes for Myc-mediated Remodeling of the PHO5 Promoter—We then looked to see whether the SAGA and SWI/SNF complexes were needed for c-Myc to remodel chromatin structure at the *PHO5* promoter. Surprisingly, deletion of *GCN5* caused only a mild reduction in chromatin remodeling (to ~80%), whereas deletion of the *SNF2* encoding a subunit of SWI/SNF had no reproducible effect at all. Furthermore, a double mutant lacking both complexes (Δ*snf2*, Δ*ada2*) was not further reduced in *PHO5* promoter remodeling compared with the single *gcn5* mutant (Fig. 5*A*). Thus, defects in chromatin remodeling of the *PHO5* promoter can only account for part of the severe defects observed for c-Myc in mutants strains defective in Gcn5-containing complexes (Fig. 4).

The results in Fig. 4, showing that the SAGA and SWI/SNF complexes are required for gene activation, were performed using a plasmid-borne reporter gene, whereas Fig. 5*A* shows the behavior at the chromosomal *PHO5* locus. Although previous analysis indicates that the *PHO5* promoter behaves similarly in both contexts (41), a formal possibility is that there are differences in the dependence of chromosomal and episomal *PHO5* promoters. To test this, we repeated the chromatin analysis on the episomal *PHO5-lacZ* reporter gene. The amount of opening measured for the plasmid-borne promoter was generally lower than for genomic *PHO5*, but comparison of the val-

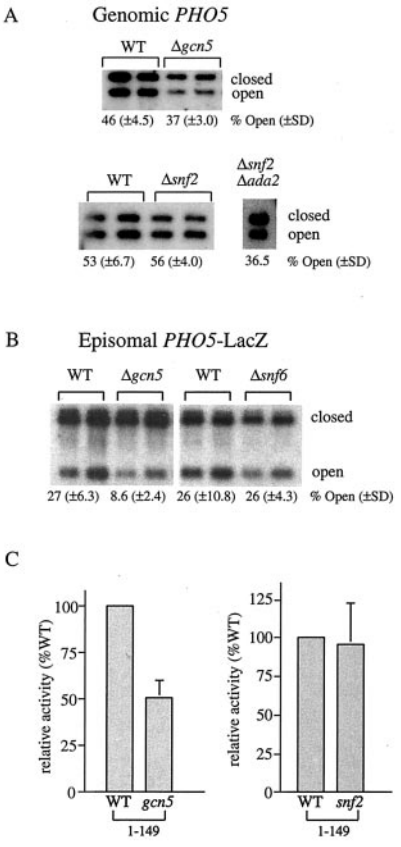


FIG. 5. **Effect of mutants defective in the SAGA and SWI/SNF complexes on c-Myc-mediated opening of the yeast *PHO5* promoter.** *A*, the ability of the 1–149P Myc-Pho4 fusion protein to remodel the genomic *PHO5* promoter in wild type and mutant strains lacking the SAGA complex (*gcn5*), the SWI/SNF complex (*snf2*), and both complexes (*snf2*, *ada2*). Annotation is as for Fig. 2*B*. *B*, the ability of the 1–149P Myc-Pho4 fusion protein to remodel the plasmid-borne *PHO5* promoter fused to the *lacZ* reporter gene in wild type, *gcn5*, and *snf6* mutant strains. Annotation is as for Fig. 2*B*. *C*, the amount of acid phosphatase induced by the 1–149P Myc-Pho4 fusion protein from the *PHO5* gene in wild type, *gcn5*, and *snf2* strains.

ues for mutant and wild type strains showed the same trend as previously. The *gcn5* mutant showed a greater reduction in opening (to ~30%), whereas no significant effect could be attributed to the SWI/SNF defect (Fig. 5*B*). Although the remodeling defect in the *gcn5* strain was increased for the plasmid-borne reporter gene, we conclude that the magnitude of the remodeling defects in the mutant strains cannot account for the severe gene activation defects measured in Fig. 4.

However, a further formal possibility was that small defects in chromatin remodeling are sufficient to cause severe defects in gene activation. To test this, we measured the activation of the genomic *PHO5* gene, encoding acid phosphatase, by measuring acid phosphatase activity. Relative to wild type strains, the levels of acid phosphatase activity were reduced to about 50% in the *gcn5* strain, and they were not significantly reduced in the *snf2* mutant (Fig. 5*C*). Thus, for the genomic *PHO5* gene, there is a very good correlation in the SAGA and SWI/SNF requirement both for remodeling of the promoter chromatin structure and for activation of the gene. Putative roles for recruited remodeling complexes at post-promoter opening steps during activation of the *PHO5-lacZ* reporter gene are discussed below.

DISCUSSION

Architecture of the c-Myc Activation Domain—As discussed in the Introduction, the c-Myc activation domain (residues 1–143) is critical for the role of c-Myc in cellular transformation during the development of cancer. Mechanistically, the activation domain functions as both an activator and a repressor of target gene expression in addition to its role in regulating the stability of the c-Myc protein itself. To understand how Myc participates in cellular transformation at the molecular level, it is necessary to characterize the contribution of regions within the activation domain to these different molecular functions. Our results in yeast clearly map the primary determinants for chromatin remodeling and gene activation to residues 1–41 and 66–127 of the c-Myc activation domain. These regions correspond to parts of the activation domain that are poorly conserved in different members of the Myc protein family. The Myc boxes that are conserved between Myc family members and which have been implicated in gene repression and proteolytic targeting of c-Myc did not have any measurable direct effect on chromatin remodeling or gene activation potential. These results are consistent with previous observations showing that the naturally occurring c-MycS form of c-Myc, which lacks most of the sequences N-terminal of Myc box II, is severely reduced in its activation potential (8).

It is more difficult to reconcile our results with previous studies showing that Myc box II plays a key role in promoter recruitment of Gcn5-containing histone acetyltransferase complexes that are normally associated with chromatin remodeling and gene activation (24). In these studies, Myc box II is clearly important for co-immunoprecipitation of c-Myc together with Gcn5 or its associated protein, TRRAP, from lysates prepared from 293 cells. To clarify the primary determinants required for protein interaction, we studied purified recombinant derivatives of the c-Myc activation domain and highly purified preparations of Gcn5-containing complexes from yeast *in vitro*. The results clearly show that Myc box II is not required for efficient interaction with the SAGA complex, which is the yeast homologue of the mammalian Gcn5 complex that is thought to interact with c-Myc. The SAGA complex is also the Gcn5 complex that functions in association with c-Myc in yeast (Fig. 4). Myc box II is, however, important for interaction with the yeast Ada complex. To our knowledge, this is the first report of an activator interacting with the Ada complex, although it is unclear whether the interaction is of functional significance *in vivo* (Fig. 4). Nonetheless, this observation indicates that Myc box II can play an important role in direct protein interactions in some cases. However, our observation that neither the NuA4 nor the SWI/SNF complex require Myc box II for efficient interaction argues that Myc box II is not generally a key part of the interaction surface. Given the previous characterization of Myc box II as a repression domain (10), an attractive possibility is that Myc box II could be a modulator domain that helps to determine whether c-Myc adopts a repressing or an activating conformation. Interestingly, a possible role of Myc box II in protein folding has been suggested previously (57). Such a model would suggest that in mammalian cell extracts the primary interaction surface of Myc box II-deleted Myc could be locked into a nonactive conformation as a result of associated proteins and/or post-translational modifications. Such a model has clear parallels to Myc box I function, which is regulated by phosphorylation (58–61), and several proteins have been identified that bind to Myc fragments containing Myc box II (57). This model is also consistent with previous reports suggesting that Myc box II is a key region for activation of c-Myc's activation potential by Ras signaling pathways (62).

We have shown that the c-Myc activation domain interacts with four well characterized chromatin-remodeling complexes from yeast. Interestingly, Myc has been shown to interact with subunits from mammalian SWI/SNF complexes previously via

its C-terminal DNA-binding/dimerization domain (63). Our observation that the activation domain interacts directly with the SWI/SNF complex combined with previous reports that it makes interactions with mammalian ATP-dependent remodeling complexes (30) suggests that Myc may make multiple interactions, at least with the ATP-dependent class of chromatin-remodeling complexes. The association of c-Myc with Gcn5 complexes was originally discovered by co-immunoprecipitation of c-Myc with the TRRAP protein. The highly conserved yeast homologue of TRRAP, Tra1, is found in both the SAGA complex and the NuA4 complex. Our observation that c-Myc interacts efficiently with the NuA4, which is otherwise thought to be structurally unrelated to the SAGA complex, is thus of interest. A potential mammalian equivalent of the NuA4 complex has been reported (64) that contains the TRRAP protein. It is thus possible that the TRRAP protein provides a surface on both this and the human SAGA complex for direct physical interaction with the c-Myc activation domain.

Our results are also of interest in relation to the nature of the interaction surface presented by the c-Myc activation domain. Many studies based on well characterized minimal activation domains have led to the view that the interacting surface of activation domains is contained within a single secondary structure element such as an α-helix (65–67). Our results clearly identify two separate regions of the c-Myc activation domain with activation potential. The strongest of these (residues 66–127) is comparable in strength with the VP16 activation domain (data not shown), but unlike previous reports for VP16 (54) we have not been able to detect significant interaction of this fragment with any of the purified chromatin-remodeling complexes studied. We have recently drawn the same conclusion from more quantitative studies of the interaction between c-Myc and the TATA-binding protein (44). Furthermore, the activation potential of both active regions in isolation is affected by mutations causing defects in the SAGA complex. Taken together, these results indicate that both the 1–41 and 66–127 regions must contribute to the interaction surface of the c-Myc activation domain and thus the interaction surface must be composed of two or more structural elements.

Role of Activator-recruited Chromatin-remodeling Complexes in Gene Activation—Chromatin-remodeling factors that are recruited to promoters by transcription factors are thought to alter the local structure of chromatin, thus modulating accessibility of other transcription factors and/or the transcriptional machinery (68). Recent reports have reported changes in the acetylation status of some promoters that are targets for activation by c-Myc during growth factor stimulation (69). In accordance with this, our results show that Gcn5 is required for optimal c-Myc-mediated chromatin opening of the endogenous *PHO5* promoter. This is reflected in a mild reduction in *PHO5* expression in *gen5* mutants. Thus, for the endogenous *PHO5* gene, remodeling of the promoter chromatin is sufficient to account for the role of Myc-recruited Gcn5 in activating gene expression. Gcn5 may play a more pronounced, transient role during initial activation of the promoter as has been recently reported for *PHO5* activation by the endogenous Pho4 transcription factor (70). Therefore, it is likely that remodeling of chromatin structure resulting from the promoter acetylation changes that have been reported (69) can account for the role of mammalian Gcn5 complexes during the activation of at least some c-Myc-regulated genes.

We have not seen any reproducible requirement for the SWI/SNF complex in Myc-Pho4-dependent activation of the endogenous *PHO5* gene. This differs from a previous report in which a weak requirement for SWI/SNF was observed for activation of *PHO5* by the endogenous Pho4 protein (71). However, as shown in the same report, the extent of the requirement for SWI/SNF depends on the potential of the activator proteins bound to the promoter. Thus, it is possible that the activation potential of the Myc-Pho4 protein, which is overexpressed in this study, exceeds that of the endogenous Pho4 protein. It is likely that SWI/SNF dependence will vary between different promoters and depending on the constellations of activator proteins with which they are associated under different physiological conditions.

Defects in promoter opening do not provide a complete explanation for the role of Gcn5-containing complexes and SWI/SNF complexes in activation of the *PHO5-lacZ* reporter gene. Activation of this gene is reduced 50-fold in *gcn5* strains, whereas chromatin opening was only reduced 3-fold. Defects in the SWI/SNF complex caused a reduction of over 5-fold in gene activation, but we were unable to show any reproducible reduction in chromatin opening at the promoter. Our results show that the different requirements for activation of endogenous *PHO5* and *PHO5-lacZ* are associated with the *lacZ* coding sequence. Thus, for the *PHO5-lacZ* gene, both Gcn5 complexes and SWI/SNF complexes appear to play a role in activation subsequent to chromatin opening of the promoter. Interestingly, it has been reported previously that in yeast, transcriptional elongation of *lacZ* reporter genes as well as some endogenous yeast genes is inefficient (72–74). These studies conclude that transcriptional elongation of long and/or GC-rich genes is inefficient and specifically dependent on a protein complex containing the Hpr1, Tho2, Mtf1, and Thp2 proteins. Our results suggest that the SAGA and SWI/SNF complexes may also play a direct role in the transcriptional elongation of a subset of genes, including *lacZ*. Consistent with this, a recent report has provided genetic evidence for coupling between the SAGA complex and transcriptional elongation (75). That report shows that mutations causing defects in the histone acetyltransferase activity of the Elongator complex show synthetic lethality with mutations defective in the SAGA complex. Since the Elongator complex becomes associated with RNA polymerase II first during elongation, this provides strong evidence for involvement of the SAGA complex in the transcriptional elongation of some important yeast mRNAs *in vivo*.

Our results suggest that whereas promoter opening by chromatin-modifying and -remodeling factors recruited by c-Myc may represent their main role during activation of some genes, they may also contribute at later steps during the activation of other genes. Consistent with this, the histone acetylation status in the promoters of some Myc target genes does not change upon mitogenic stimulation, leading to the conclusion that recruited HATs must function later in gene activation (32). Whereas direct support for such a role will require extensive further study, there is already substantial supportive evidence in the literature. For example, artificial activator proteins have been shown to work at the elongation level previously (76), and human SWI/SWF complex has been implicated in efficient elongation through transcriptional pause sites *in vitro* (77). The same function has recently been reported for the highly conserved Elongator histone acetyltransferase complex (75). Furthermore, Myc target genes including the ornithine decarboxylase gene and the *myc* gene itself, have been shown to be regulated at the level of transcriptional elongation (78–82). Finally, the transcriptional attenuation of *myc* gene elongation seen in normal cells is abrogated in Burkitt's lymphoma cells, clearly implicating regulation of elongation as an important component in the ontogeny of cancer (83).

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