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Histone acetyltransferase activity and interaction with ADA2 are critical for GCN5 function *in vivo*

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Yeast GCN5 is one component of a putative adaptor complex that includes ADA2 and ADA3 and functionally connects DNA-bound transcriptional activators with general transcription factors. GCN5 possesses histone acetyltransferase (HAT) activity, conceptually linking transcriptional activation with enzymatic modification at chromatin. We have identified the minimal catalytic domain within GCN5 necessary to confer HAT activity and have shown that *in vivo* activity of GCN5 requires this domain. However, complementation of growth and transcriptional activation in *gcn5*$^-$ cells required not only the HAT domain of GCN5, but also interaction with ADA2. The bromodomain in GCN5 was dispensable for HAT activity and for transcriptional activation by strong activators; however, it was required for full complementation in other assays. Fusion of GCN5 to the bacterial lexA DNA binding domain activated transcription *in vivo*, and required both the HAT domain and the ADA2 interaction domain. These results suggest that both functions of GCN5, HAT activity and interaction with ADA2, are necessary for targeting and acetylation of nucleosomal histones.

Keywords: adaptor/GCN5/genetics/histone acetyltransferase/transcription

Introduction

Activation of transcription by RNA polymerase II requires several classes of proteins that function in a coordinate manner (Tjian and Maniatis, 1994). General factors constituting the basal transcription machinery recognize the core promoter composed of the TATA box and adjacent initiation site. They include RNA polymerase II and other factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH) required for initiation and elongation of mRNA (for reviews, see Zawel and Reinberg, 1993, 1995; Buratowski, 1994). Transcriptional activators bind to specific DNA sequences upstream of core promoters (Ptashne, 1986, 1988; Goodrich *et al.*, 1996) and increase the rate of transcription by the basal machinery.

Mechanisms of activation are not fully understood, although it is generally accepted that proteins distinct from general factors and activators play a role (Guarente, 1995). One class of proteins, often referred to as adaptors, mediators or co-activators (Berger *et al.*, 1990; Kelleher *et al.*, 1990; Pugh and Tjian, 1990), has been identified in different laboratories by genetic and biochemical methods (Dynlacht *et al.*, 1991; Meisterernst *et al.*, 1991; Berger *et al.*, 1992; Luo *et al.*, 1992; Chrivia *et al.*, 1993; Kim *et al.*, 1994; Koleske and Young, 1994). These cofactors facilitate transcription, possibly by promoting interactions between transcriptional activators and the general transcriptional machinery (Lewin, 1990; Ptashne and Gann, 1990; Roeder, 1991; Gill and Tjian, 1992), although their precise mechanisms of action may vary.

Co-activators were identified originally as TATA box binding protein (TBP)-associated factors (TAFs). Although TBP is sufficient for basal transcription, TAFs are required to activate transcription *in vitro* (Dynlacht *et al.*, 1991). Other co-activators interact with specific activators and potentiate activation *in vivo*. For example, CREB binding protein (CBP) binds directly and specifically to the phosphorylated form of CREB, and has been shown to potentiate transcription of a variety of activators (Chrivia *et al.*, 1993; Kwok *et al.*, 1994). Oct co-activator from B cells (OCAB) is a co-activator for Oct-1 that stimulates the activity of natural immunoglobulin promoters (Luo and Roeder, 1995; Strubin *et al.*, 1995).

A genetic selection in yeast identified proteins that functionally interact with the activation domain of the herpes simplex virus activator, VP16 (Berger *et al.*, 1992). Several genes, *ADA2* (Berger *et al.*, 1992), *ADA3* (Piña *et al.*, 1993), *GCN5* (Marcus *et al.*, 1994) and *ADA5* (Marcus *et al.*, 1996; Roberts and Winston, 1996) were cloned, and mutations in any of them slowed yeast growth and reduced transcriptional activation by some acidic activators, such as VP16 and yeast GCN4, but had little effect on other activators, such as yeast HAP4. ADA2 physically interacted with activation domains derived from VP16 (Silverman *et al.*, 1994; Barlev *et al.*, 1995) and GCN4 (but not HAP4) (Barlev *et al.*, 1995), and also with TBP (Barlev *et al.*, 1995). ADA2, ADA3 and GCN5 interacted with each other *in vitro* (Horiuchi *et al.*, 1995) and *in vivo* (Candau and Berger, 1996), which argued strongly for the existence of a physiologically relevant ADA complex. Taken together, these data suggest that the ADA complex bridges interactions between specific activation domains and the general factors.

To activate transcription *in vivo*, the transcriptional machinery must overcome repression caused by association of genes with nucleosomes, which requires chromatin reorganization (for a review, see Grunstein, 1990; Wolffe, 1994b; Struhl, 1996). Genetic approaches in yeast have identified transcriptional regulators that appear to have evolved to deal with the repressive environment of chromatin. For example, the SWI–SNF complex alters chromatin structure (Hirschhorn *et al.*, 1992) and is required to enhance transcription by many transcriptional

activators (Peterson and Herskowitz, 1992; Laurent *et al.*, 1993).

In addition, the adaptor GCN5 has been shown to possess histone acetyltransferase (HAT) activity (Brownell *et al.*, 1996). Since hyperacetylation of amino-terminal tails of core histones correlates with the activity of certain genes (Csordas, 1990; Loidl, 1994; Wolffe, 1994a; Wolffe and Pruss, 1996), the HAT activity of GCN5 suggests a link between nucleosome acetylation and transcriptional activation. Further evidence of the role of histone acetylation and deacetylation in the regulation of transcription in eukaryotes is the isolation of a mammalian histone deacetylase (Taunton *et al.*, 1996), related to the yeast transcriptional regulator Rpd3p (Vidal and Gaber, 1991).

Recombinant GCN5 is able to acetylate histone H3 when present in a mixture of 'free' histones, but is unable to acetylate histones in nucleosomes (Kuo *et al.*, 1996; Yang *et al.*, 1996). Complexes containing GCN5 in both *Tetrahymena* (J.Brownell and C.D.Allis, unpublished data) and yeast (P.Grant and J.Workman, personal communication) acetylate core histones in nucleosomes. One explanation for this difference is that additional components of the multi-subunit ADA complex are required for GCN5 to acetylate physiologically relevant, nucleosomal substrates. Indeed, here we show that sequences within GCN5 required for interaction with ADA2 were necessary for *in vivo* function of GCN5 in all assays tested. We also identify the GCN5 domain necessary to confer HAT activity *in vitro*, and show that this minimal catalytic domain is required for growth and transcriptional activation *in vivo*. These results directly link the HAT domain to transcriptional activation *in vivo* and provide genetic evidence that the ADA complex is required for GCN5 to acetylate nucleosomal substrates *in vivo*.

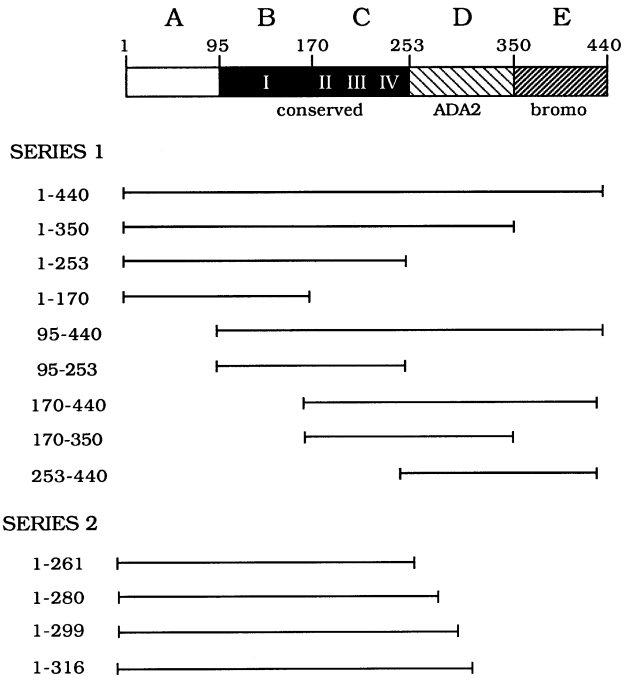


Fig. 1. Schematic of GCN5 deletion derivatives. GCN5 was divided into five regions based on the degree of conservation between yeast and human GCN5 (see text). (A) The non-conserved region, (B) the conserved region between amino acids 95 and 170, (C) the highly conserved region between amino acids 170 and 253, (D) the domain of interaction with ADA2 and (E) the bromodomain motif are indicated. Roman numerals in the conserved domain refer to putative catalytic regions I–IV as described in Brownell *et al.* (1996). Series 1: amino-termini and carboxy-termini deletions of GCN5 (Candau and Berger, 1996). Full-length GCN5 comprises amino acids 1–440. Deleted versions of GCN5 are composed of residues: 1–350, 1–253, 1–170, 95–253, 95–440, 170–350, 170–440 and 254–440 which are shown relative to full-length. Series 2: carboxy-termini deletions of GCN5. Deleted versions of GCN5 are composed of residues 1–261, 1–280, 1–299 and 1–316.

Results

Mapping of the HAT domain in vitro

Yeast GCN5 was divided into five subregions (Figure 1, top) based on the degree of conservation between GCN5 homologs identified in organisms ranging from yeast to humans, as follows: (A) the amino-terminus (amino acids 1–95) is poorly conserved; (B) amino acids 95–170 are well conserved (64% similarity); (C) the region between amino acids 170 and 253 is highly conserved (88% similarity); (D) the region between amino acids 253 and 350 is well conserved (55% similarity) and contains the region necessary for interaction with ADA2 (Candau *et al.*, 1996); and (E) the bromodomain (aa 350–440) has 57% similarity and is present in a variety of eukaryotic proteins having putative co-activator or adaptor function (Haynes *et al.*, 1992). We have argued previously, based solely upon amino acid conservation between the yeast and *Tetrahymena* enzyme, that the region between amino acids 120 and 253 may constitute the enzymatic HAT domain, and have identified four extremely highly conserved subregions (I–IV) (Brownell *et al.*, 1996). We have also proposed that the bromodomain may target HAT activity to chromatin 'receptors' (Brownell and Allis, 1996), since yeast HAT1 (Kleff *et al.*, 1995), the cytoplasmic HAT, does not contain a bromodomain, although it does contain other sequence elements, presumably catalytic, in common with GCN5 (Brownell *et al.*, 1996).

A series of deletions mutants which progressively delete from the amino- or carboxy-terminus of GCN5 have been described (Candau and Berger, 1996; Figure 1, series 1), based on the conservation described above. To identify the region possessing HAT activity *in vitro*, each of the deletion mutants was subcloned into a bacterial expression plasmid, in-frame with a 'six-his' tag. Protein was induced, purified on nickel–agarose beads and similar amounts of each protein (Figure 2A) were separated on SDS–PAGE, polymerized in the presence of free histones for an 'in-gel' HAT assay, as previously described (Brownell and Allis, 1995).

Deletion of the first 95 amino acids ($GCN5_{95–440}$), or the last 90 amino acids, including the bromodomain ($GCN5_{1–350}$), had little effect on HAT activity (Figure 2B and C) as compared with the wild-type enzyme prepared under identical conditions. Deletion of the amino-terminal 170 amino acids ($GCN5_{170–440}$, $GCN5_{170–350}$) or the carboxy-terminal 190 amino acids ($GCN5_{1–253}$) reduced HAT activity to approximately one-quarter of that of the full-length protein. Deletion of both ends ($GCN5_{95–253}$) reduced activity to 10% of wild-type. Thus, loss of sequences including the HAT subregion I (aa 120–140; Figure 1) reduced activity significantly, but not completely. Mutants containing deletions between amino acids 170 and 253 ($GCN5_{254–440}$ and

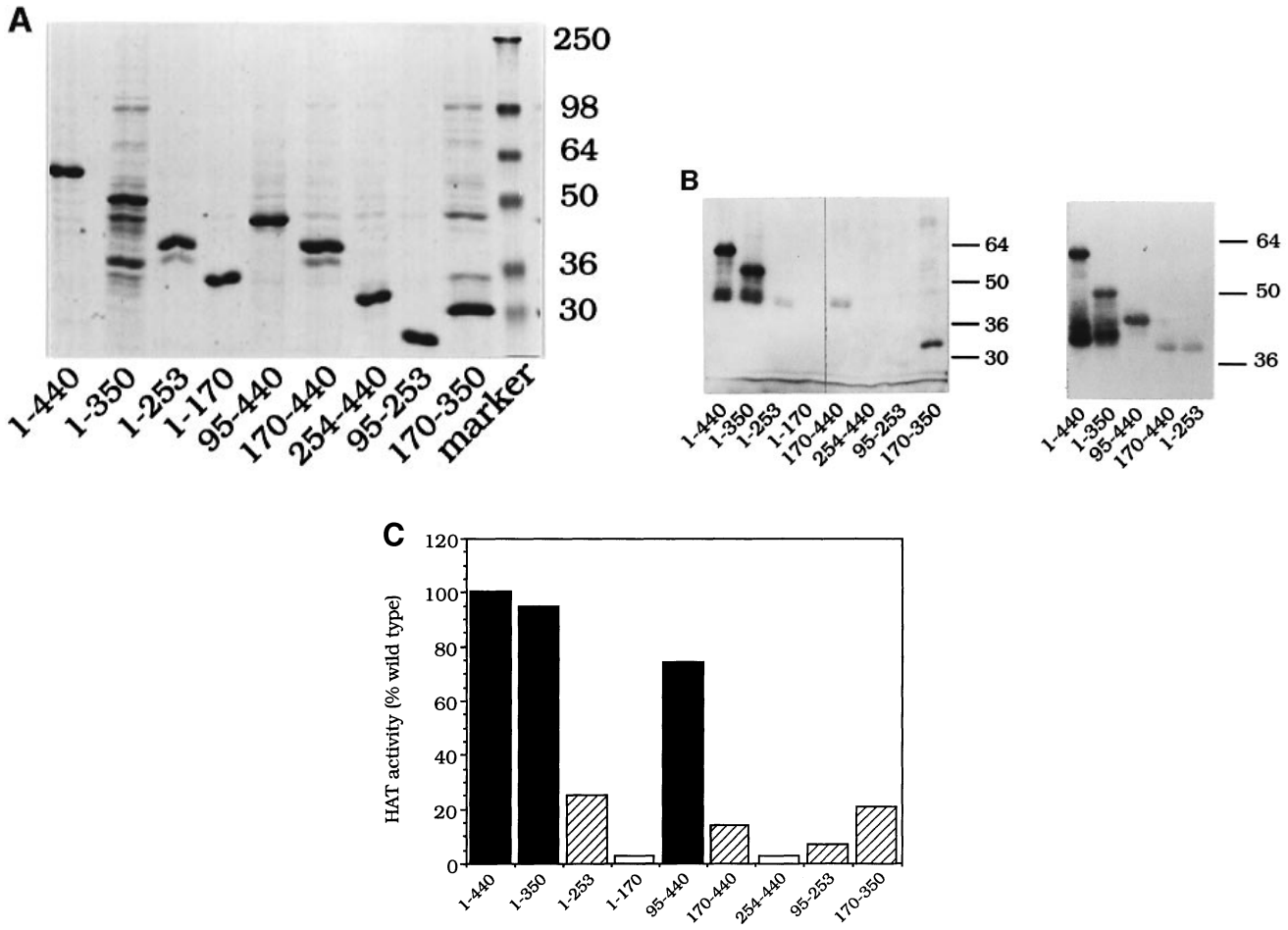


Fig. 2. HAT activity of the deletion mutants of GCN5 (series 1). (**A**) Coomassie Blue staining of the recombinant deletion peptides. Crude bacterial extracts were purified through Ni^{2+}-NTA–agarose beads and a sample from each preparation was electrophoresed on 8% SDS–PAGE and stained with Coomassie Blue. Protein size standards are shown on the right. (**B**) In-gel histone acetyltransferase assay of the deletion peptides. Purified proteins (as shown in A) were analyzed by 8% SDS–PAGE and assayed for HAT activity as described previously (Brownell and Allis, 1995). (**C**) Histogram showing quantification of the HAT assay. Signals from the autoradiogram in (B) were quantified by densitometry and normalized to the amount of protein in (A). Values relative to the full-length protein are shown.

$GCN5_{1–170}$), had <3% HAT activity compared with the full-length protein, and thus defined a minimal HAT domain, between 170 and 253.

Internal deletions of each HAT subregion I–IV, between amino acids 120 and 253 (Figure 1), were negative for HAT activity (data not shown). However, these proteins failed to interact with ADA2 (data not shown), even though the region of interaction for ADA2 as previously determined (aa 254–350; Candau and Berger, 1996) was present. Thus, these mutant derivatives were probably folding incorrectly, and were not studied further.

Separation of the HAT domain from the ADA2 interaction domain in vitro

The above data suggest that the minimal HAT domain of GCN5 mapped between amino acids 170 and 253, while the domain possessing full HAT activity mapped between 95 and 350. We wished to map the carboxy-terminal border of the HAT domain more precisely to better delineate and ideally separate the region of HAT activity from the region of interaction with ADA2. Therefore, we prepared a second series of deletion mutants with carboxy-terminal endpoints between 253 and 350 (Figure 1, series 2).

First, we tested the ability of each of the series 2 mutants to acetylate histones *in vitro* (Figure 3). A similar amount of each protein (Figure 3A) was compared with the full-length GCN5 or with $GCN5_{1–253}$ in the in-gel assay (Figure 3B and C). As before, $GCN5_{1–253}$ had <25% of wild-type activity, but the next largest peptide, $GCN5_{1–261}$, had activity comparable with wild-type. Each of the other, even larger peptides (aa 1–280, 1–299 and 1–316) also possessed 'wild type' HAT activity. Thus, the carboxy-terminal border of the HAT domain was defined by these mutants at amino acid 261.

Next, we determined the ability of the above (series 2) mutants to interact with ADA2 using *in vitro* co-immunoprecipitation, which previously was used to define the region of interaction with ADA2 between amino acids 254 and 350 of GCN5 (Candau and Berger, 1996). Each mutant was co-translated *in vitro* with full-length ADA2. All of the GCN5 mutants larger than 1–261 ($GCN5_{1–280}$, $_{1–299}$ and $_{1–316}$) were immunoprecipitated using α-ADA2 antibody (Figure 4). However, $GCN5_{1–261}$ did not co-precipitate with ADA2, indicating that the domain of

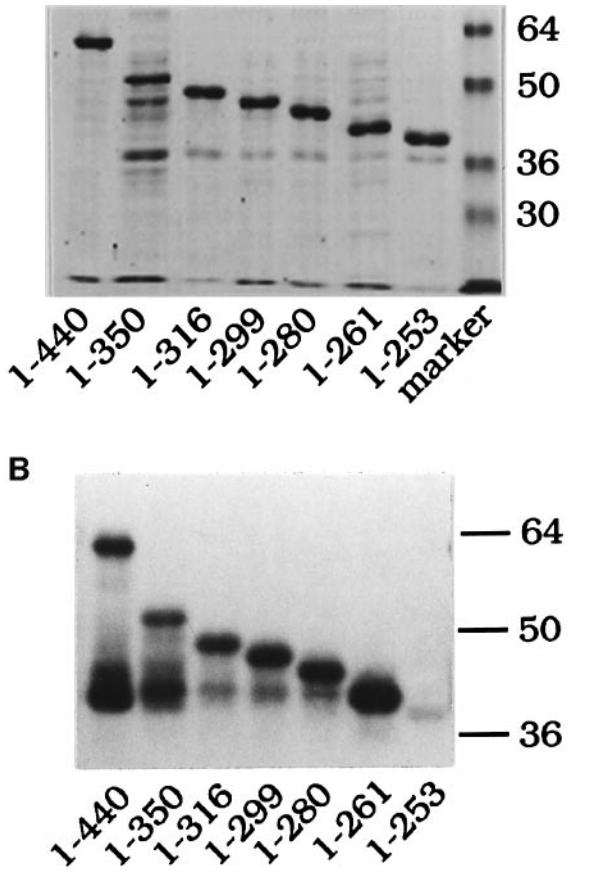


Fig. 3. HAT activity of the deletion mutants of GCN5 (series 2). (**A**) Coomassie Blue staining of the recombinant deletion peptides. Crude bacterial extracts were purified through Ni^{2+}-NTA–agarose beads and a sample from each preparation was electrophoresed on 8% SDS–PAGE and stained with Coomassie Blue. Protein size standards are shown on the right. (**B**) In-gel histone acetyltransferase assay of the deletion peptides. Purified proteins (as shown in A) were analyzed by 8% SDS–PAGE and assayed for HAT activity as described previously (Brownell and Allis, 1995). (**C**) Histogram showing quantification of the HAT assay. Signals from the autoradiogram in (B) were quantified by densitometry and normalized to the amount of protein in (A). Values relative to the full-length protein are shown.

ADA2 interaction is contained between amino acids 254 (Candau and Berger, 1996) and 280 of GCN5. Since the HAT activity displayed by $GCN5_{1–261}$ was comparable with full-length GCN5, the lack of $GCN5_{1–261}$ interaction with ADA2 was not due to inappropriate folding. These results distinguished the carboxy-terminal border of the HAT domain (aa 261) from the carboxy-terminal border of the ADA2 interaction domain (aa 280).

Domains of GCN5 required for in vivo growth complementation in the gcn5⁻ strain

Three distinct functional regions have been identified within GCN5. We have defined here the boundaries of the HAT domain and the ADA2 interaction domain *in vitro*, and the bromodomain has been shown to be required for full function of GCN5 *in vivo* (Marcus *et al.*, 1994). We wished to determine whether the HAT or other regions of GCN5 are required for *in vivo* function of GCN5.

Genetic deletion of *GCN5* resulted in defective colonial growth on minimal synthetic media (Marcus *et al.*, 1994). Thus, the ability of the deletion mutants to complement

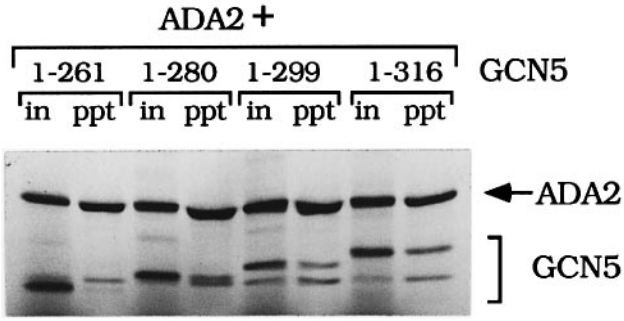


Fig. 4. Co-immunoprecipitations of ADA2 and GCN5 deletion mutants. Each GCN5 deletion mutant (series 2) was co-translated *in vitro* with ADA2 and immunoprecipitated with α-ADA2 antisera. ^{35}S-labeled proteins were visualized by autoradiography after 12% SDS–PAGE. in = input and ppt = precipitate. The side arrow indicates ADA2 protein and the bracket indicates GCN5 deletion peptides. Note the presence of a non-specific protein that migrates between $GCN5_{1–260}$ and $GCN5_{1–280}$, which is likely to be an ADA2 degradation product.

growth of a *GCN5* disruption strain was tested. The mutants were cloned into a yeast expression vector, and each one was transformed and restreaked onto minimal media. Wild-type GCN5 or vector alone served as positive and negative controls for growth in this assay (Figure 5A).

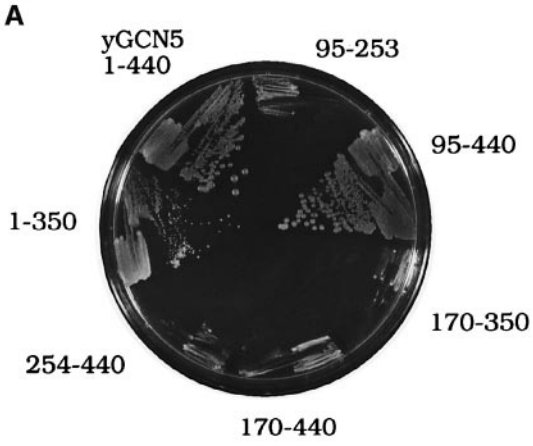
The only deletion that maintained full growth complementation was $GCN5_{95–440}$, which lacked the amino-terminal 95 amino acids. All other deletions resulted in complete or partial loss of growth complementation (Figure 5A). Deletion of the conserved subregion I (Figure 1) of the HAT domain ($GCN5_{170–440}$) resulted in loss of growth complementation, which may be caused by the significant loss (80% reduced) of HAT activity *in vitro* (Figure 2C). Mutant $GCN5_{1–261}$ complemented growth very poorly, despite having full HAT activity *in vitro* (Figure 3C) and, interestingly, this mutant was unable to interact with ADA2 *in vitro* (Figure 4). Partial complementation was seen in each mutant lacking sequences distal to amino acid 280 (Figure 5A).

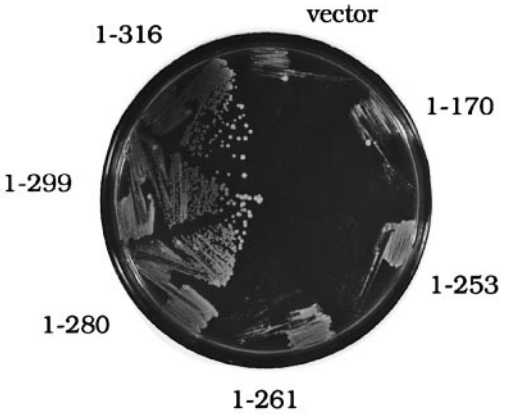
As previously shown, the deletion of the bromodomain ($GCN5_{1–350}$) caused partial loss of growth complementation in the *GCN5* deletion strain (Marcus *et al.*, 1994; Figure 5A). Surprisingly, this mutant complemented growth more poorly than the smaller peptides $GCN5_{1–280}$, $_{1–299}$ or $_{1–316}$, which is shown more clearly in the liquid growth assay (Figure 5B). Immunoblot analysis of the mutants containing these deletions revealed that all were comparable in stability with wild-type, with the exception of the bromodomain deletion, which was partially unstable (data not shown). (Note that the strain used differed from those used in previous studies; Marcus *et al.*, 1996.) However, the partial instability of $GCN5_{1–350}$ did not seem to account entirely for its poor growth complementation, since the same mutant was indistinguishable from wild-type in other *in vivo* assays (see below).

Overall, these data indicate that critical regions of GCN5 for growth complementation lie between amino acids 95 and 280.

Domains of GCN5 required for complementation of GAL4–VP16-mediated growth inhibition in the gcn5⁻ strain

We previously have shown that overexpression of GAL4–VP16 (Sadowski *et al.*, 1988), a chimeric activator com-

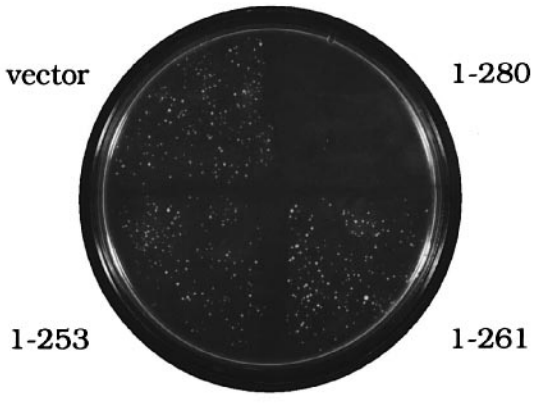




B

GCN5 deletion mutant	generation time (hours)
1-440	3.20
1-350	4.20
1-316	3.55
1-299	3.75
1-280	3.75
1-261	4.25
1-253	4.25
vector	4.70

Fig. 5. Growth complementation of *GCN5* deletion mutants in the *gcn5*$^-$ strain. (**A**) Each *GCN5* deletion mutant was transformed into the *gcn5*$^-$ strain, restreaked onto minimal synthetic media and grown at room temperature for 4 days. Full-length *GCN5* (1–440) and vector alone were used as positive and negative controls. (**B**) Generation time of the *GCN5* deletion mutants. The second series of the *GCN5* deletion mutants was transformed into the *gcn5*$^-$ strain and each transformant was inoculated into liquid minimal synthetic media and rotated at 25°C. Aliquots of each mutant were taken every 2 h and the OD of the cultures was measured. The generation time was calculated as the OD doubling time during exponential growth. Full-length *GCN5* (1–440) and vector alone were used as positive and negative controls.



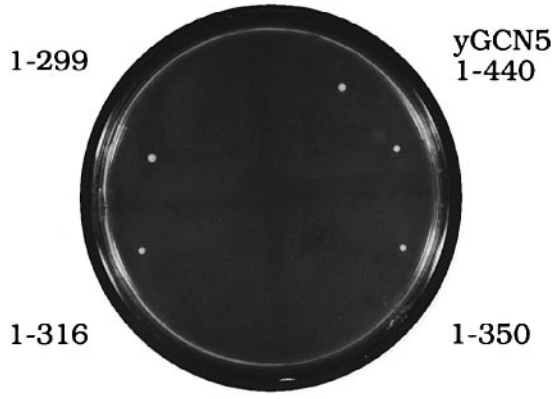


Fig. 6. Growth inhibition of GAL4–VP16 in the presence of the *GCN5* deletion mutants. The ability of the second series of the *GCN5* deletion mutants to confer the slow growth phenotype in the presence of high copy plasmid expressing GAL4–VP16 is shown. *GAL4–VP16* was co-transformed with the indicated *GCN5* mutants into the *gcn5*$^-$ strain. Transformants were plated onto minimal synthetic media and were grown at 30°C for 4 days. Full-length *GCN5* (1–440) and vector alone were used as positive and negative controls.

posed of the GAL4 DNA binding domain and the transcriptional activation domain derived from the herpes simplex virus protein, VP16, results in strong growth inhibition in cells containing wild-type GCN5, and this inhibition is relieved when GCN5 is deleted (Marcus *et al.*, 1994). Growth inhibition by GAL4–VP16 may be caused by sequestration of essential transcription factors (Gill and Ptashne, 1988) by the potent VP16 activation domain, and was used as the basis for the genetic screen (Berger *et al.*, 1992) that led to the identification of adaptors ADA2, ADA3, GCN5 and ADA5. We used this assay to study the effects of the deletion derivatives of GCN5.

A high copy yeast expression plasmid that over-produced GAL4–VP16 was co-transformed with each of the deletion mutants into the *gcn5*$^-$ strain and the transformants were plated onto minimal medium (Figure 6). Wild-type GCN5 or vector alone were used as positive and negative controls for growth inhibition caused by GAL4–VP16. The results were largely consistent with the growth complementation assay. $GCN5_{95–440}$ was similar

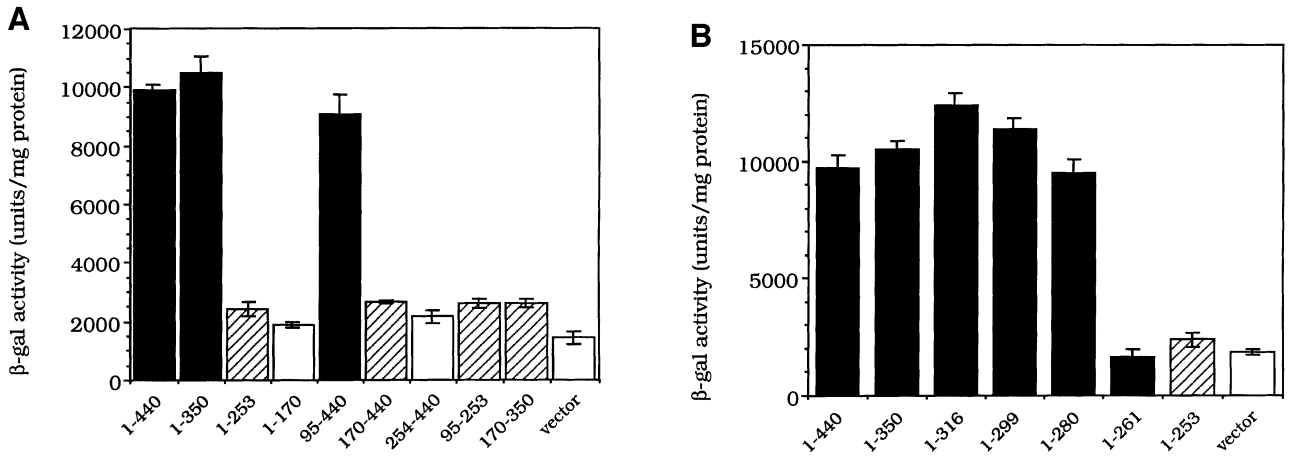


Fig. 7. Transcriptional activation in the presence of the GCN5 deletion mutants by the strong activator GAL4–VP16. The series 1 (**A**) and 2 (**B**) of *GCN5* deletion mutants were co-transformed into PSY316Δ*gcn5*, along with low copy plasmids expressing *GAL4–VP16* and reporter pLGSD5 (Guarente *et al.*, 1982), containing bacterial *lacZ* driven by the *GAL1–10* promoter. β-Gal activity was determined as units per mg of protein. Error bars represent the standard error about the mean from at least two independent experiments.

to wild-type in both assays (data not shown). Mutants that lacked portions of the HAT domain ($GCN5_{170–440}$ or $GCN5_{254–440}$) were unable to restore toxicity by GAL4–VP16 (data not shown), just as they were unable to complement growth. All of the deletions carboxy-terminal to amino acid 280 ($GCN5_{1–280}$, $_{1–299}$, $_{1–316}$ and $_{1–350}$) complemented growth inhibition, while $GCN5_{1–261}$ and $GCN5_{1–253}$ did not (Figure 6), indicating that interaction with ADA2 is necessary for functional interaction with GAL4–VP16, just as it was necessary for complementation of growth.

The bromodomain mutant, $GCN5_{1–350}$, behaved differently in each growth assay. In the GAL4–VP16 inhibition assay, the mutant was indistinguishable from wild-type (Figure 6). In contrast, the mutant only partially complemented growth and, in fact, complemented more poorly than shorter mutants (Figure 5A and B). This dual behavior was seen in other experiments, as described below.

Domains of GCN5 required for complementation of transcriptional activation in the gcn5⁻ strain

We wished to identify domains of GCN5 required for transcriptional activation *in vivo* and, in particular, whether the HAT domain had a critical role in transcriptional activation. Activation by GAL4–VP16, containing the full-length VP16 activation domain (aa 413–490) (Triezenberg *et al.*, 1988), previously was shown to be reduced 7- to 10-fold in strains deleted for *ADA*s (Berger *et al.*, 1992; Piña *et al.*, 1993), including *GCN5* (Marcus *et al.*, 1994). We tested the ability of each GCN5 deletion mutant to complement transcriptional activation mediated by GAL4–VP16 in a *gcn5⁻* background.

A low copy plasmid expressing GAL4–VP16 was co-transformed with a reporter containing bacterial lacZ driven by GAL4 binding sites, and wild-type GCN5 or the deletion mutants. The results of β-gal assays from the first series of deletion mutants is shown in Figure 7A. The $GCN5_{1–350}$ and $GCN5_{95–440}$ deletion mutants complemented the *gcn5⁻* strain, since they exhibited levels of transcription similar to wild-type GCN5. The other mutants, which contained further amino- or carboxy-terminal deletions of GCN5 ($GCN5_{1–253}$, $_{1–170}$, $_{170–440}$ and $_{254–440}$) were unable to complement. These results paralleled those obtained in the HAT assay, since the relative profiles of activation *in vivo* (Figure 7A) and HAT activity *in vitro* (Figure 2C) were similar, suggesting that HAT activity is necessary for transcriptional activation.

As before in the growth assay, the relative contributions of HAT and ADA2 interaction could not be determined, since both functions were reduced in the $GCN5_{1–253}$ mutant. Thus, the second series of mutants was tested in the GAL4–VP16 transcription assay. Parallel to the growth assay, loss of ADA2 interaction resulted in a completely defective protein ($GCN5_{1–261}$), even though it possessed full HAT activity *in vitro* (Figure 3B and C). Thus the HAT domain, on its own, was not sufficient for *in vivo* activity either in growth complementation or in transcriptional activation, and ADA2 interaction was also required.

One difference between the growth assay and the transcription assay was the effect of deleting the carboxy-terminus to amino acid 280. These mutants ($GCN5_{1–350}$, $_{1–316}$, $_{1–299}$ and $_{1–280}$), which were similar to wild-type for activation by GAL4–VP16 (Figure 7B), were partially defective in growth, especially the bromodomain deletion (Figure 5). Failure to detect intermediate phenotypes in the transcription assay could be explained by the potency of the full-length VP16 activation domain, which could mask partial defects. In this case, weaker activation domains might reveal intermediate phenotypes of the GCN5 mutants.

To test this hypothesis, complementation of GAL4–$VP16_{470–490}$ (Barlev *et al.*, 1995) was assayed with each of the deletion mutants. Previous results showed that this region of VP16 constitutes an activation subdomain, displaying 3- to 4-fold weaker activation than the full-length VP16, but normal interaction with ADA2 *in vitro* (Barlev *et al.*, 1995). Indeed, GAL4–$VP16_{470–490}$ required GCN5 function *in vivo*, since its activity was reduced nearly 10-fold in the *gcn5⁻* strain (Figure 8A). As was observed with the full-length VP16 activation domain, the deletions $GCN5_{1–261}$ and $GCN5_{1–253}$ were completely defective. In contrast to the complete complementation by the intermediate peptides with full-length VP16, each peptide ($GCN5_{1–350}$, $_{1–316}$, $_{1–299}$ and $_{1–280}$) exhibited partial

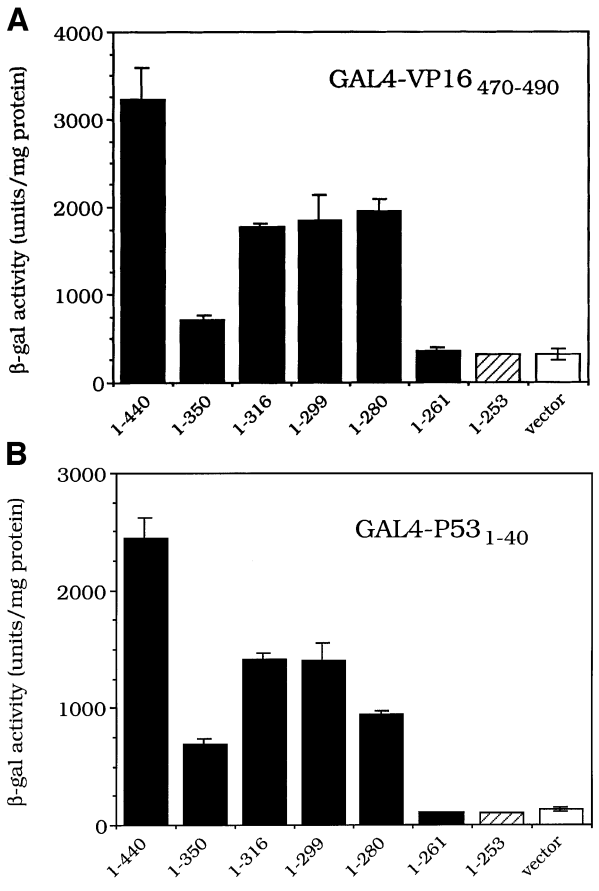


Fig. 8. Transcriptional activation by weak activators in the presence of the GCN5 deletion mutants. (**A**) GAL4–VP16$_{470–490}$ transcriptional activation in the presence of the GCN5 deletion mutants. The series 2 of *GCN5* deletion mutants were co-transformed into the *gcn5*$^-$ strain, along with low-copy plasmid expressing GAL4–VP16$_{470–490}$ and reporter pLGSD5. β-Gal activity was determined as units per mg of protein. Error bars represent the standard error about the mean from at least two independent experiments. (**B**) GAL4–p53$_{1–40}$ transcriptional activation in the presence of the GCN5 deletion mutants. The series 2 of *GCN5* deletion mutants were co-transformed into PSY316Δ*gcn5*, along with plasmids expressing low-copy GAL4–p53$_{1–40}$ and reporter pLGSD5. β-Gal activity was determined as units per mg of protein. Error bars represent the standard error about the mean from at least two independent experiments.

complementation, and, as in the growth complementation, the bromodomain mutant was more defective than the shorter peptides.

A second chimeric activator was also tested, GAL4–p53, that contained the amino terminal 1–40 amino acids of the p53 activation domain (Fields and Jang, 1990; Farmer *et al.*, 1992; Scharer and Iggo, 1992) fused to GAL4. GAL4–p53 activation dropped ~20-fold in the *gcn5*$^-$ strain (R.Candau and S.L.Berger, submitted). GAL4–p53$_{1–40}$ showed the same profile of dependence on GCN5 as did GAL4–VP16$_{470–490}$, with intermediate complementation by the shorter peptides GCN5$_{1–316}$, $_{1–299}$ and $_{1–280}$, and even poorer complementation by GCN5$_{1–350}$.

Overall, these results demonstrate that the HAT domain, as well as the ADA2 interaction domain, are critical for GCN5′s role in transcriptional activation. Furthermore, the carboxy-terminal region of GCN5, beyond the ADA2 interaction domain, was required for full activation by weaker transcriptional activators, but not a strong activator.

Both the HAT and ADA2 interaction domains are required for lexA–GCN5 activity in vivo

The presence of the intrinsic HAT activity in GCN5 raises the question of whether the HAT catalytic domain can activate transcription if it is artificially targeted to a promoter, thus rendering it activation domain-independent. If the roles of activators and the ADA complex are to 'deliver' the HAT to promoters, then fusion of the HAT domain to a DNA binding domain should result in a protein capable of transcriptional activation. In contrast, if either activator or the ADA complex is required to acetylate nucleosomal histones, then additional domains may be required in addition to the HAT catalytic domain. To distinguish between these alternatives, full-length *GCN5* or various *GCN5* deletion mutants were genetically fused to the lexA DNA binding domain (Figure 9A). These were then transformed into yeast, and their ability to activate a lacZ reporter driven by lexA binding sites was determined.

LexA–GCN5 activated transcription 10-fold better than lexA alone (Figure 9B). The HAT domain was required for lexA–GCN5 activity, since deletion of it (lexA–GCN5$_{254–440}$) lowered activation nearly to background levels. However, lexA–GCN5$_{1–261}$, containing only the HAT domain, was unable to activate. The addition of the ADA2 interaction domain allowed lexA–GCN5$_{1–280}$ to activate, in fact, 2-fold better than the full-length GCN5 fusion. The requirement for lexA–GCN5 to associate with ADA2 was also shown by complete loss of activity in *ada2*$^-$ cells (Figure 9C). The level of lexA–GCN5 protein was comparable in *ADA2*$^+$ and *ada2*$^-$ cells. Collectively, these results suggest that transcriptional activation with either intact GCN5 and activators, or with lexA–GCN5, requires that several conditions be met. Association with promoter sequences, catalytic function provided by the HAT domain and interaction with ADA2 all seem to be required for high levels of transcriptional activation.

Discussion

Yeast GCN5 was originally identified as a regulatory factor required for function of the yeast activator GCN4 (Georgakopoulos and Thireos, 1992), and was isolated independently as a factor necessary for maximal transcriptional activation by GAL4–VP16 (Marcus *et al.*, 1994). GCN5 interacts with a second factor, ADA2, which was isolated in the same genetic selection (Berger *et al.*, 1992; Marcus *et al.*, 1994). Recently, GCN5 has been shown to possess histone acetyltransferase activity *in vitro* (Brownell *et al.*, 1996), potentially linking transcriptional activation with the covalent modification of the amino-termini of histones. In the present study, we have mapped the boundaries of the HAT domain of GCN5, and have shown that this domain is required for activity *in vivo* in several independent assays. Our data suggest that the HAT domain, while essential for activation *in vivo*, is not sufficient. Rather, the ADA2 interaction domain in GCN5 is also required for full activity.

Critical elements of the HAT catalytic domain lie between amino acids 170 and 250

Mapping the HAT catalytic domain *in vitro* indicates that the full domain is encompassed within amino acids 95–

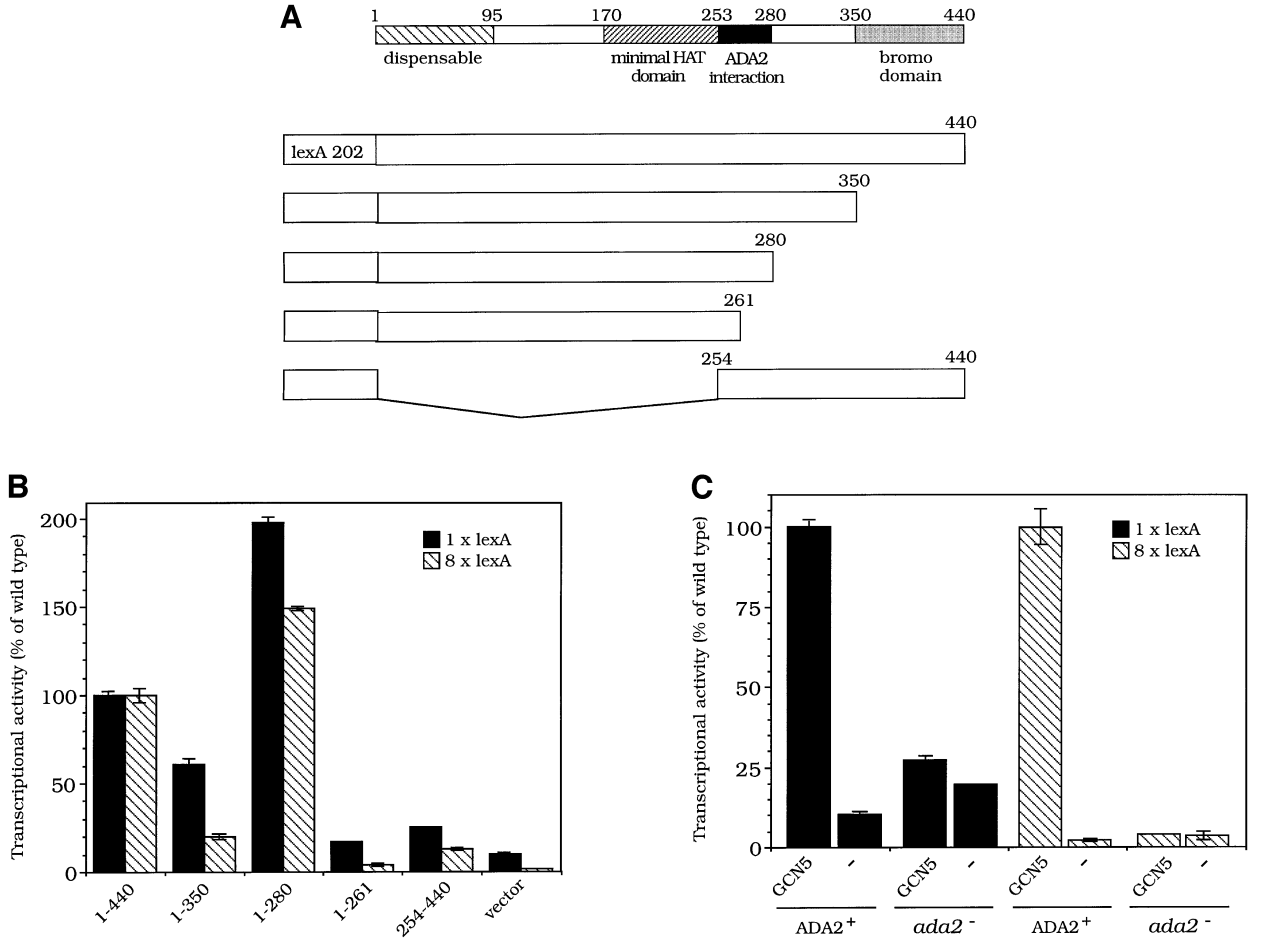


Fig. 9. Transcriptional activation by lexA–GCN5 deletion mutants. (**A**) Schematic of the lexA–GCN5 deletion mutants. The bacterial lexA DNA binding domain was fused to full-length GCN5 and to the deletion mutants $GCN5_{1–280}$, $_{1–261}$, $_{1–299}$, $_{1–350}$ and $_{254–440}$. The domains of full-length GCN5 are shown above. (**B**) Transcriptional activation by lexA–GCN5. The indicated lexA–GCN5 deletion mutants, or lexA alone as negative control, were co-transformed in the *gcn5*$^-$ strain along with a lexA reporter containing either one (YEP21) (Brent and Ptashne, 1985) or eight lexA binding sites (Candau *et al.*, 1996). β-Gal activity is shown relative to full-length GCN5. Error bars represent the standard error about the mean from two independent experiments. (**C**) Transcriptional activation by lexA–GCN5 in the *ada2*$^-$ strain. Full-length lexA-GCN5 or lexA alone as negative control were co-transformed in PSY316 or PSY316Δ*gcn5* along with a lexA reporter containing either one or eight lexA binding sites. β-Gal activity is shown relative to full-length GCN5. Error bars represent the standard error about the mean from two independent experiments.

261, while a minimal domain lies between 170 and 253 (Figure 10). These data support the notion that the most conserved portions of GCN5 (aa 120–253) constitute the HAT domain (Brownell *et al.*, 1996). We have suggested previously that His145 may comprise an essential residue of the active site. Since the minimal domain lies between 170 and 253, critical active site residues will probably lie within these boundaries.

Critical elements for in vivo activation lie between amino acids 95 and 280

In contrast to the mapping of the HAT domain (aa 95–261), an extended region of GCN5 was required for full *in vivo* function. A 20 amino acid region (to aa 280) beyond the carboxy-terminal boundary of the HAT domain (at aa 261) was crucial for growth and transcriptional activation by both GAL4–VP16 and GAL4–p53. Since immunoprecipitation of GCN5 by ADA2 also required sequences up to amino acid 280, ADA2 interaction appears to be absolutely necessary for GCN5 function *in vivo*. Since GCN5 and ADA2 appear to be two components of an ADA complex including ADA3 (Horiuchi *et al.*, 1995;

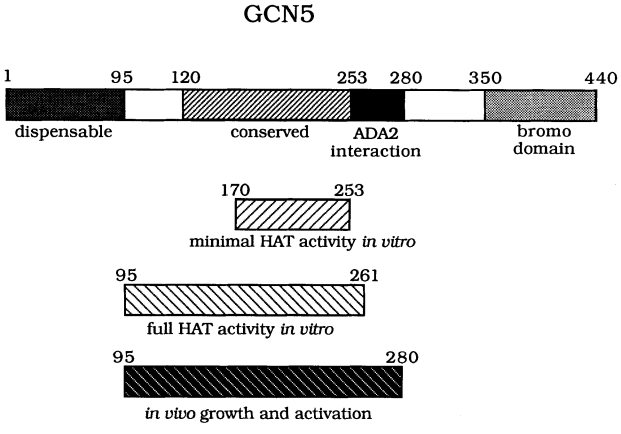


Fig. 10. Function of the GCN5 domains. Schematic of full-length GCN5 is shown above, and below are shown the domains necessary to confer minimal HAT activity *in vitro*, full HAT activity *in vitro* and complementation of growth and transcriptional activation *in vivo*.

Candau and Berger, 1996) and ADA5 (Marcus *et al.*, 1996), it is likely that GCN5 requires the ADA complex to acetylate nucleosomal histones and, thus, to activate transcription *in vivo*. Whether acetylation of nucleosomal substrates occurs through subunit exchange of GCN5/ADA subunits with defined sub-nucleosomal subunits, as has been proposed recently (Roth and Allis, 1996), remains unknown, but is consistent with the involvement of ADA2 reported here.

Elements between amino acids 95 and 170 that contribute to HAT activity also appeared to be crucial for function, since $GCN5_{170–440}$ (which had only 20% HAT activity) lacked the ability to function *in vivo*. Consistent with this result is the finding that the point mutation H145A in GCN5 reduces HAT activity 2- to 5-fold in all our *in vitro* assays (J.Z. and C.D.Allis, unpublished data). Thus, either GCN5 is extremely sensitive to partial loss of HAT activity or additional functions localize to amino acids 95–170.

The bromodomain was required for weak activator function in vivo, but not for strong activator function

Previous studies have shown an important role for the bromodomain of GCN5 *in vivo* (Marcus *et al.*, 1994). Since HAT1, a cytoplasmic histone acetyltransferase, does not contain a bromodomain (Kleff *et al.*, 1995), one possibility is that the bromodomain is required for access of GCN5 to nucleosomal histones in the nucleus. However, the present study indicates that the bromodomain is not likely to be a critical component in the acetylation of nucleosomal histones. First, the bromodomain was completely dispensable for full HAT activity *in vitro* and activation by the strong activator GAL4–VP16. Second, weak activators required the bromodomain for full activity, but were less affected by bromodomain deletion compared with deletion of the HAT domain. Finally, lexA–$GCN5_{1–280}$, which lacked the bromodomain, was a stronger activator than full-length lexA–GCN5. Taken together, these data suggest that the bromodomain is not involved directly in activation through nucleosomal histone acetylation, but may play a critical role in protein–protein interactions that are not detected in these assays. For example, we have detected an interaction between the bromodomain of human GCN5 (Candau *et al.*, 1996) and the p70 subunit of Ku autoantigen (N.Barlev and S.L.Berger, unpublished data). The Ku autoantigen p70–p80 heterodimer is the DNA binding component of the DNA-PK holoenzyme (for a review, see Jackson, 1996). This interaction may be regulatory, as DNA-PK was found to phosphorylate and inhibit the HAT activity of human GCN5 *in vitro*. A putative yeast homolog of Ku interacted with yeast GCN5 (N.Barlev and S.L.Berger, unpublished data), suggesting a conserved regulatory function of the bromodomain.

Finally, we observed in several assays, including growth complementation, weak activator function and lexA–GCN5 activity, that the bromodomain deletion mutant $GCN5_{1–350}$ was less active than shorter mutants ($GCN5_{1–316}$, $GCN5_{1–299}$ and $GCN5_{1–280}$). This may indicate the presence of a repression domain with a carboxy-terminal border in the region 315–350, although the partial instability of $GCN5_{1–350}$ *in vivo* may explain these results. However, the stronger activity of lexA–$GCN5_{1–280}$ compared with full-length lexA–GCN5 is consistent with the presence of a repression domain.

Targeting of GCN5 to a promoter, as well as interaction with ADA2, is required for function in vivo

We reasoned that if GCN5, and therefore the HAT domain, requires targeting to promoters via interaction with DNA-bound transcriptional activators (Wolffe and Pruss, 1996), fusion of GCN5 to a DNA binding domain should be active independently of interaction with *bona fide* activators. Indeed, lexA–GCN5 activated transcription, and this activation required the HAT domain of GCN5. This is consistent with previous observations that activators associate with components of the ADA complex to target the complex to promoter regions (Silverman *et al.*, 1994; Barlev *et al.*, 1995).

Furthermore, if the sole role of the ADA complex is to provide appropriate surfaces for protein–protein interaction with activation domains, then lexA–GCN5 should not require ADA2 or its ADA2 interaction domain for activity *in vivo*. Since lexA–GCN5, in the absence of ADA2, and lexA–$GCN5_{1–261}$, lacking the ability to interact with ADA2, were both inactive, the ADA complex apparently has an additional function *in vivo* beyond interaction with activation domains.

Why do both GCN5 and lexA–GCN5 require interaction with ADA2, and presumably the ADA complex, for function *in vivo*? Since recombinant GCN5 acetylates 'free' histones, but not nucleosomal histones *in vitro* (Kuo *et al.*, 1996; Yang *et al.*, 1996), it is likely that the ADA complex contributes critical determinants for interaction with histones in their native nucleosomal state. Indeed, in both yeast (P.Grant and J.Workman, personal communication) and *Tetrahymena* (J.Brownell and C.D.Allis, unpublished data) GCN5 is one component of multi-subunit complexes which are capable of acetylating nucleosomal histones. Further mutagenesis of GCN5, combined with analysis of native complexes, will clarify the mechanism of acetylation of nucleosomal substrates.

Materials and methods

Yeast strains

The *trp1* derivatives of PSY316Δ*ada2* (*MAT*α *ade2-101* Δ*his3-200 leu2-3,112 lys2 ura3-52 trp1*) and PSY316Δ*gcn5* (*MAT*α *ade2-101* Δ*his3-200 leu2-3,112 lys2 ura3-52 trp1*) have been described previously (Candau *et al.*, 1996).

Plasmids and deletion mutants

Plasmids were constructed using standard procedures (Ausubel *et al.*, 1994).

To generate the deletion mutants of *GCN5* for *in vitro* translation, fragments comprising residues 1–261, 1–280, 1–299 and 1–316 of *GCN5* bearing a *Not*I restriction site at the 5′ end and an *Eag*I restriction site at the 3′ end were amplified by PCR. These fragments were digested with *Eag*I and inserted in SP64-*Not*I (Candau and Berger, 1996) opened with *Not*I.

For protein expression, pRSETB-*Not*I was generated by cloning a *Not*I linker in pRSETB (Invitrogen) digested with *Pvu*II. The *GCN5* deletion mutants were isolated from the corresponding SP64-*Not*I-*GCN5* digested with *Eag*I and then cloned into pRSETB-*Not*I as six-histidine protein fusions.

To generate the TRP1 yeast expression vector pPC87, the linker AAGCTTGTCGACCCCGGGGAATTCAGATCTCTGCAGGCGGC-CGC was inserted into pPC97 (Chevray and Nathans, 1992) opened

with *Hin*dIII–*Not*I. This plasmid (pPC98) was then digested with *Bam*HI–*Apa*I and the fragment containing the ADH promoter and terminator was then cloned into pPC86 (Chevray and Nathans, 1992) opened with *Bam*HI–*Apa*I.

The *GCN5* deletion mutants were cloned into pPC87 by digestion of each SP64-*Not*I-*GCN5* deletion mutant with *Eag*I. Each *GCN5* fragment was then inserted into pPC87 opened with *Not*I.

To generate the *lexA* fusions to the *GCN5* deletion mutants, SP64-*Not*I-*GCN5*$_{1–440}$, $_{1–261}$, $_{1–280, 1–350}$ and $_{254–440}$ were digested with *Eag*I, and the corresponding *GCN5* fragments were inserted into pRS316-*lexA* (Piña *et al.*, 1993) digested with *Not*I.

GAL4–VP16 expression vectors were described by Berger *et al.* (1992).

Growth complementation, growth inhibition and β-galactosidase assays

PSY316Δ*gcn5* was transformed as described (Ito *et al.*, 1983) with the *GCN5* deletion mutants and plated in fully supplemented SD medium. After 3 days, single colonies were streaked on SD minimal medium and incubated at 30°C or at room temperature, and their ability to complement growth was tested.

Alternatively, single colonies were inoculated into liquid SD minimal medium and rotated at 30°C overnight. The cultures were then diluted to an OD of 0.02, rotated at 25°C and the OD was checked every 2 h up to a total of 20 h.

Growth inhibition by GAL4–VP16 was carried out in PSY316Δ*gcn5* co-transformed with the *GCN5* deletion mutants and GAL4–VP16 high expression plasmid, as described (Berger *et al.*, 1992).

β-Gal assays (Rose *et al.*, 1988) were carried out in PSY316Δ*gcn5* or PSY316Δ*ada2*Δ*gcn5* transformed with the plasmids described for each experiment. β-Gal activity was determined as units per mg of protein.

The lacZ reporters used were pLGSD5 (Guarente *et al.*, 1982) for GAL4–VP16 activation, and YEp21 (one lexA binding site) (Brent and Ptashne, 1985) and LexA-8x (eight lexA binding sites) (Candau *et al.*, 1996) for lexA–GCN5 activation.

Protein expression

The *GCN5* deletion mutants were transformed into *Escherichia coli* strain JM109 and induced with IPTG and M13/T7 phage according to XPRESS SYSTEM (Invitrogen). yGCN5 peptides were isolated under denaturing conditions and purified (based on XPRESS SYSTEM, Invitrogen) on Ni-NTA–agarose (Qiagen). Briefly, cells were solubilized in 0.1 M sodium phosphate, 10 mM Tris–HCl pH 8, 6 M guanidine-HCl, and then applied onto Ni^{2+}-NTA resin. The resin was washed initially with 0.1 M sodium phosphate, 10 mM Tris–HCl pH 8, 8 M urea and then with 0.1 M sodium phosphate, 10 mM Tris–HCl pH 6.3, 8 M urea. His-tagged GCN5 proteins were eluted in 0.1 M sodium phosphate, 10 mM Tris–HCl pH 4.5, 8 M urea.

HAT assay and analysis of histone acetylation

GCN5 deletions purified as described above were loaded onto 8% SDS–PAGE and the gels were stained with Coomassie Blue to normalize the amount of proteins.

HAT activity assays were performed as described by Brownell and Allis (1995). Briefly, samples were loaded onto an 8% SDS–PAGE gel containing 1 mg/ml calf thymus histones (Sigma). Following electrophoresis, gels were washed for 1 h at room temperature in buffer A [50 mM Tris–HCl pH 8, 1 mM dithiothreitol (DTT), 0.1 M EDTA] containing 20% (v/v) isopropanol, they were then incubated in buffer A containing 8 M urea for 1 h and then overnight at 4°C in buffer A with 0.04% Tween-40. The gels were washed in buffer B [50 mM Tris–HCl pH 8, 10% (v/v) glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 M EDTA] prior to 2 h incubation at 30°C with [^{3}H]acetyl-CoA (5 μCi/3 ml buffer B). The labeled gels were washed with 5% trichloroacetic acid and fluorographed.

HAT activity was quantified using a densitometer (Hewlett Packard Scan-Jet II cx/T).

In vitro translation and co-immunoprecipitation assays

In vitro translation and co-immunoprecipitation experiments were performed as described by Candau *et al.* (1996).

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