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A Multiplicity of Coactivators Is Required by Gcn4p at Individual Promoters In Vivo

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Transcriptional activators interact with multisubunit coactivators that modify chromatin structure or recruit the general transcriptional machinery to their target genes. Budding yeast cells respond to amino acid starvation by inducing an activator of amino acid biosynthetic genes, Gcn4p. We conducted a comprehensive analysis of viable mutants affecting known coactivator subunits from the *Saccharomyces* Genome Deletion Project for defects in activation by Gcn4p in vivo. The results confirm previous findings that Gcn4p requires SAGA, SWI/SNF, and SRB mediator (SRB/MED) and identify key nonessential subunits of these complexes required for activation. Among the numerous histone acetyltransferases examined, only that present in SAGA, Gcn5p, was required by Gcn4p. We also uncovered a dependence on CCR4-NOT, RSC, and the Paf1 complex. In vitro binding experiments suggest that the Gcn4p activation domain interacts specifically with CCR4-NOT and RSC in addition to SAGA, SWI/SNF, and SRB/MED. Chromatin immunoprecipitation experiments show that Mbf1p, SAGA, SWI/SNF, SRB/MED, RSC, CCR4-NOT, and the Paf1 complex all are recruited by Gcn4p to one of its target genes (*ARG1*) in vivo. We observed considerable differences in coactivator requirements among several Gcn4p-dependent promoters; thus, only a subset of the array of coactivators that can be recruited by Gcn4p is required at a given target gene in vivo.

Eukaryotic activator proteins stimulate transcription by binding to their target genes and carrying out two general functions: (i) altering the locations or structures of nucleosomes and (ii) recruiting TATA-binding protein (TBP), other general transcription factors (GTFs), and RNA polymerase II (RNA PolII) to the promoter. Most activators carry out these functions indirectly by recruiting multisubunit complexes, collectively called coactivators (39, 70, 90). One class of coactivators uses ATP hydrolysis to displace nucleosomes and thereby expose or obscure protein binding sites in the promoter (91, 124). Each of the nucleosome remodeling complexes of *Saccharomyces cerevisiae*, known as SWI/SNF, RSC, ISW1, and ISW2, contains a different subunit harboring the ATPase activity of the complex (reviewed in references 70 and 91). Although each has been implicated in transcriptional activation in vivo (5, 38, 51, 85, 123), only the nonessential SWI/SNF complex has been shown to interact physically with activators (93, 139) and be recruited to a promoter for nucleosome remodeling and transcriptional activation in vitro (45, 96, 139). Recruitment of the SWI/SNF complex by yeast activators has also been demonstrated in living yeast cells by chromatin immunoprecipitation (ChIP) assays (24, 126).

Another class of coactivators alters chromatin structure by acetylation of lysines in the amino-terminal tails of histones. This modification destabilizes higher-order chromatin structure (116) and also may stimulate binding of other coactivator proteins containing a bromodomain (9, 91, 120, 135). The SAGA complex is the best-characterized yeast coactivator in this class (109, 118), and its histone acetyltransferase (HAT) subunit, Gcn5p, acetylates nucleosomal H3 and H2B (43). SAGA also binds to TBP in vitro (8, 119) and can promote TBP recruitment in vivo (31). Purified SAGA interacts with several yeast activators (30, 42, 92) and can be recruited to a chromatin template for transcriptional stimulation in a HAT-dependent manner (132). Activator recruitment of Gcn5p HAT activity or SAGA subunits was also demonstrated in vivo (12, 31, 63, 66).

SAGA shares a subset of TBP-associated factors (TAFs) with the general transcription factor TFIID, which also recruits TBP to certain promoters (104). All but one of the non-TAF subunits of SAGA are dispensable (109, 118); however, the majority of TAFs are essential proteins (84, 103). Transcriptome analysis indicates that SAGA and TFIID have redundant coactivator functions (67). Recruitment of TBP by the activator Gcn4p appears to involve the single polypeptide Mbf1p, which serves as an adaptor between the DNA binding domain of Gcn4p and TBP (127).

Other high-molecular-weight HAT complexes in yeast include NuA4, which acetylates predominantly nucleosomal H4, and NuA3, which acetylates exclusively nucleosomal H3 (42). NuA4 contains only one known nonessential subunit, Eaf3p (4, 33), which is dispensable for acetylation by NuA4 in vitro but required for wild-type (WT) basal expression of *PHO5*, *HIS4*, and *TRP4* in vivo. These data indicate a role for NuA4 in transcription, as suggested by its interactions with activators in vitro (42, 132). The NuA3 complex, in contrast, has displayed no activator interactions, and mutation of its HAT subunit, Sas3p (57), seems to affect only silenced chromatin (105).

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A third class of coactivators, of which the best characterized example is SRB mediator (SRB/MED), interacts with RNA PolII and the GTFs TFIIF and TFIIB (13, 89). The Srb subunits of SRB/MED interact genetically with the C-terminal domain (CTD) of the largest subunit of RNA PolII (128). Many SRB/MED subunits have been implicated in positive or negative transcriptional control (13, 89). Most notably, Gal11p, Med2p, Pgd1p, and Sin4p seem to comprise a subcomplex (27, 88) that is required for SRB/MED-dependent transcriptional activation (44, 71, 88) and for activator binding to SRB/MED in vitro (98). SRB/MED associates specifically with the CTD nonphosphorylated form of RNA PolII and is absent from the phosphorylated elongating enzyme (102, 125). Thus, SRB/MED may function primarily in recruitment of nonphosphorylated RNA PolII to the promoter.

A distinct RNA PolII holoenzyme has been identified (114, 133) that contains TFIIF and TFIIB, the SRB/MED subunits Rgr1p, Sin4p, and Gal11p (70), and other subunits specific to this form of holoenzyme, including Paf1p and Cdc73p (17, 86, 114, 117). Although this Paf1 complex associates with nonphosphorylated RNA PolII and contains GTFs, its genetic and physical interactions with Spt4p-Spt5p and the Spt16p-Pob3p-containing CP complex suggest a role in transcriptional elongation (117). Consistent with the latter, Paf1p was found to be associated with the transcribed regions as well as promoters of several yeast genes in vivo (102). Furthermore, the Paf1 complex interacts with Hpr1p, a stoichiometric component of the THO/TREX complex that was implicated in transcriptional elongation and transcription-induced recombination (3, 19, 121).

The Paf1 complex also interacts with Ccr4p, a protein with positive and negative regulatory functions in gene expression (17, 25, 74). A large fraction of the Ccr4p resides in a 1.2-MDa complex, distinct from the Paf1 complex, containing Caf1p/Pop2p, Caf40p, Caf130p, and the five NOT proteins (21, 74). *NOT1* to *NOT5* were identified genetically as negative regulators that prevent transcription from the noncanonical TATA element at *HIS3* in the absence of Gcn4p (Not$^-$ phenotype) (23). Consistent with a role in transcriptional repression, mutations in *NOT1*, *NOT3*, *NOT5*, and *CAF1* can suppress the lethal phenotype of an *srb4* mutation (69). However, mutations in *CCR4*, *CAF1*, *NOT2*, and *NOT3* impair *CYC1* derepression in nonfermentable carbon sources, implicating these subunits in gene activation (74). Numerous other proteins are associated with the core CCR4-NOT complex, including a subset of SRB proteins associated with RNA PolII holoenzyme (75). The CCR4-NOT complex has been shown to interact with TBP and certain TAFs, most likely in the context of TFIID, possibly to inhibit TFIID binding to nonconsensus TATA elements (6, 73).

Gcn4p is a transcriptional activator of amino acid, vitamin, and purine biosynthetic genes in yeast (94) and is induced at the translational level by starvation for any amino acid (49). Gcn4p function is dependent on clusters of bulky hydrophobic residues distributed throughout its acidic activation domain (29, 54). Because of their functional redundancy, multiple hydrophobic clusters must be mutated simultaneously to impair Gcn4p function in vivo and abolish its interactions with coactivators in vitro (30). Gcn4p binds in vitro to SAGA, SWI/SNF, SRB/MED, and NuA4 (30, 42, 92, 93, 96, 132), and mutations were isolated in subunits of the first three complexes that decrease activation by Gcn4p in vivo (10, 40, 93, 96, 100). There is also ChIP evidence that Gcn4p recruits SWI/SNF (126) and Gcn5p HAT activity (63, 64) to target promoters in vivo.

In the present study, we conducted a comprehensive analysis of Gcn4p coactivator requirements by testing viable mutants from the *Saccharomyces* Genome Deletion Project for defects in activation by Gcn4p in vivo. Our results confirm previous findings that Gcn4p function requires SAGA, SWI/SNF, and SRB/MED and also reveal a dependence on CCR4-NOT, RSC, and the Paf1 complex for full activation by Gcn4p in vivo. In vitro binding experiments suggest that Gcn4p interacts specifically with CCR4-NOT and RSC, in addition to SAGA, SWI/SNF, and SRB/MED. ChIP assays indicate that SAGA, SWI/SNF, SRB/MED, CCR4-NOT, RSC, the Paf1 complex, and Mbf1p are recruited to the *ARG1* promoter by Gcn4p. Although Gcn4p can recruit an array of coactivators to the same promoter in vivo, not all of these factors are required for WT activation at every Gcn4p target gene.

MATERIALS AND METHODS

Yeast strains, genetic methods, and plasmids. All strains were grown at 25°C. Strains from the *Saccharomyces* Genome Deletion Project were purchased from Research Genetics, and most are listed in Table 1 (exceptions are noted below). Strain LSO2 was generated from a cross of strain 13701 (BY4742 *med2*Δ) with strain 5489 (BY4741 *med1*Δ). HAT double mutants were generated by crossing strains 17285 (BY4742 *gcn5*Δ) or 14518 (BY4742 *nut1*Δ) with strains 2742 (BY4741 *elp3*Δ), 1551 (BY4741 *ayt1*Δ), 5608 (BY4741 *hpa2*Δ), 308 (BY4741 *hpa3*Δ), 6568 (BY4741 *sas2*Δ) or 4518 (BY4741 *nut1*Δ).

All strains used for ChIP analysis containing Myc_{13}-tagged alleles of coactivator genes were created in the *gcn4*Δ strain (strain 249) by a PCR-based method for tagging chromosomal genes by yeast transformation (77). The pFA6a-13Myc-His3MX6 plasmid was used as a template, and transformants were selected on synthetic complete medium lacking histidine (SC−His medium). His$^+$ colonies were analyzed by colony PCR to verify the presence of the tag in the gene of interest and by Western analysis to verify expression of the tagged protein. Colony PCRs were performed as described previously (2). Strains used for glutathione *S*-transferase (GST) pull-down experiments containing Myc_{13}-tagged alleles of coactivator genes were created in the same manner in the WT strain BY4741.

Plasmids containing *CAF1*, *PAF1*, *SRB10*, *NOT5*, or *SWI3* were made by PCR amplification of the relevant gene followed by restriction digestion and insertion of the fragments into YCplac33 for *CAF1* and *PAF1* (41) or pRS316 for *SRB10*, *NOT5*, and *SWI3* (115). Plasmids p2382 and pHQ1239, encoding HA_3-Gcn4p, were produced as follows. Plasmid pCD48 (29) contains a *GCN4* allele with a *Bgl*II site just before the stop codon. Into this site, a *Bgl*II fragment encoding three tandem hemagglutinin (HA) epitopes was cloned to generate plasmid p2382. A *Sal*I-*Eco*RI fragment from p2382 containing *GCN4-HA$_3$* was subcloned into the high-copy-number *URA3* vector YEplac195 (41) to generate pHQ1239. Plasmid pSK-1 containing *GCN4-Myc$_{13}$* was created by inserting a *Bgl*II-*Bam*HI fragment encoding 13 Myc epitopes into the *Bgl*II site of plasmid pCD48.

For reporter gene assays, deletion mutants and the isogenic WT and *gcn4*Δ strains were transformed with pHYC2 carrying the *UAS$_{GCRE}$-CYC1-lacZ* reporter (50) or pKN7 carrying the *HIS3-GUS* fusion (92). Three independent transformants were replica plated to sulfometuron methyl (SM)-containing medium and compared to untransformed cells to ensure that the SM^s phenotype was unchanged. All strains were grown to saturation in SC−Ura medium and diluted to an optical density at 600 nm of ~0.5 in two identical cultures. After 2.5 h of growth, one set of cultures was harvested and resuspended in SC medium lacking Ile and Val and also lacking uracil (SC−ILV−Ura medium) containing 0.5 μg of SM per ml. Uninduced cultures were grown for a total of 6 h, and induced cultures were grown in the presence of SM for 6 h. Enzyme assays were performed as previously described for β-galactosidase (83) and β-glucuronidase (92).

GST pull-down, Western blot, and Northern blot analyses. Bacterial extracts containing GST proteins were prepared from transformants of *Escherichia coli* strain BL21 (30). Yeast whole-cell extracts (WCEs) were prepared (138) and

TABLE 1. SM resistance and UAS_{GCRE}-*CYC1-lacZ* expression phenotypes of cofactor mutants

Name	Parent[a]	Relevant genotype[b]	Cofactor complex	SM resistance (% of WT)[c]	β-Gal activity (% of WT) for[d]:	
					Uninduced UAS_{GCRE}-*CYC1-lacZ*	Induced UAS_{GCRE}-*CYC1-lacZ*
BY4741	NA	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	NA[e]	100	100	100
BY4742	NA	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	NA			
BY4743	NA	*MAT***a**/*MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ura3Δ0/ura3Δ0*	NA	100	100	100
249	BY4741	*gcn4*Δ::*kanMX4*	Activator	5	10	1.0
30249	BY4743	*gcn4*Δ::*kanMX4*/*gcn4*Δ::*kanMX4*	Activator	5	1.1	0.2
6753	BY4741	*mbf1*Δ::*kanMX4*	NA	100		
7335	BY4741	*mbf1*Δ::*kanMX4*	NA	100	92	180
1799	BY4741	*ahc1*Δ::*kanMX4*	ADA	100	86	145
4282	BY4741	*ada2*Δ::*kanMX4*	ADA, SAGA	51	54	**22***
3534	BY4741	*ada3*Δ::*kanMX4*	ADA, SAGA	46	86	**30***
7285	BY4741	*gcn5*Δ::*kanMX4*	ADA, SAGA	41	46	**29**
1038	BY4741	*ada1*Δ::*kanMX4*	SAGA	26	8.8	**5.1**
7309	BY4741	*ada5*Δ::*kanMX4*	SAGA	26	13	**3.1***
4228	BY4741	*spt3*Δ::*kanMX4*	SAGA	89	*330*	100
3218	BY4741	*spt7*Δ::*kanMX4*	SAGA	24	100	**25***
2666	BY4741	*spt8*Δ::*kanMX4*	SAGA	95	*380*	110
184	BY4741	*yer049*Δ::*kanMX4*	NuA3	100	110	**59**
2150	BY4741	*yp1101*Δ::*kanMX4*	NuA3	100	*340*	*220*
3078	BY4741	*sas3*Δ::*kanMX4*	NuA3	100	67	68
7143	BY4741	*eaf3*Δ::*kanMX4*	NuA4	100	28	100
5308	BY4741	*bdf1*Δ::*kanMX4*	TFIID	62	74	65
3767	BY4741	*bdf2*Δ::*kanMX4*	TFIID	100	100	140
2742	BY4741	*elp3*Δ::*kanMX4*	HAT	89		
1551	BY4741	*ayt1*Δ::*kanMX4*	HAT	100		
5608	BY4741	*hpa2*Δ::*kanMX4*	HAT	100		
308	BY4741	*hpa3*Δ::*kanMX4*	HAT	100		
6568	BY4741	*sas2*Δ::*kanMX4*	HAT	100		
1114	BY4741	*rpd3*Δ::*kanMX4*	HDAC	100		
5347	BY4741	*hda1*Δ::*kanMX4*	HDAC	91		
5487	BY4741	*hos1*Δ::*kanMX4*	HDAC	100		
4561	BY4741	*hos2*Δ::*kanMX4*	HDAC	100		
2136	BY4741	*hos3*Δ::*kanMX4*	HDAC	100		
3738	BY4741	*sir2*Δ::*kanMX4*	HDAC	100		
1760	BY4741	*hst1*Δ::*kanMX4*	HDAC	100		
2813	BY4741	*hst2*Δ::*kanMX4*	HDAC	100		
1801	BY4741	*hst3*Δ::*kanMX4*	HDAC	100		
3550	BY4741	*hst4*Δ::*kanMX4*	HDAC	100		
2123	BY4741	*tfg3*Δ::*kanMX4*	Multiple[f]	28	40	**22**
1586	BY4741	*swi2*Δ::*kanMX4*	SWI/SNF	35	*200*	**40***
1250	BY4741	*swi3*Δ::*kanMX4*	SWI/SNF	62	170	**33***
7175	BY4741	*snf5*Δ::*kanMX4*	SWI/SNF	55	120	**28***
6409	BY4741	*snf6*Δ::*kanMX4*	SWI/SNF	55	110	**27***
4008	BY4741	*snf11*Δ::*kanMX4*	SWI/SNF	103	140	**61**
15398	BY4742	*swp73*Δ::*kanMX4*	SWI/SNF	41	160	**39***
4686	BY4741	*rsc1*Δ::*kanMX4*	RSC	92	140	100
5266	BY4741	*rsc2*Δ::*kanMX4*	RSC	70	45	42
3385	BY4741	*isw1*Δ::*kanMX4*	ISW1	100		
1601	BY4741	*isw2*Δ::*kanMX4*	ISW2	100		
4500	BY4741	*itc1*Δ::*kanMX4*	ISW2	100		
6160	BY4741	*chd1*Δ::*kanMX4*	Homodimer	100		
1431	BY4741	*not3*Δ::*kanMX4*	CCR4-NOT	100	70	140
207	BY4741	*not4*Δ::*kanMX4*	CCR4-NOT	100	150	100
5491	BY4741	*not5*Δ::*kanMX4*	CCR4-NOT	42	56	**17***
7123	BY4741	*caf1*Δ::*kanMX4*	CCR4-NOT	27	44	**8.2***
7048	BY4741	*caf4*Δ::*kanMX4*	CCR4-NOT	97	100	110
5647	BY4741	*caf16*Δ::*kanMX4*	CCR4-NOT	100	62	110
6925	BY4741	*caf17*Δ::*kanMX4*	CCR4-NOT	91	*280*	80

Continued on following page

TABLE 1—*Continued*

Name	Parent[a]	Relevant genotype[b]	Cofactor complex	SM resistance (% of WT)[c]	β-Gal activity (% of WT) for[d]: Uninduced *UAS$_{GCRE}$-CYC1-lacZ*	β-Gal activity (% of WT) for[d]: Induced *UAS$_{GCRE}$-CYC1-lacZ*
1156	BY4741	*caf40*Δ::*kanMX4*	CCR4-NOT	100	130	120
6405	BY4741	*caf130*Δ::*kanMX4*	CCR4-NOT	100	71	110
6990	BY4741	*dbf2*Δ::*kanMX4*	CCR4-NOT	70	46	93
3858	BY4741	*dhh1*Δ::*kanMX4*	CCR4-NOT	64	11	**6.3**
4279	BY4741	*srb9*Δ::*kanMX4*	SRB/MED, CCR4-NOT	84	25	69
2786	BY4741	*srb10*Δ::*kanMX4*	SRB/MED, CCR4-NOT	58	170	**35***
5351	BY4741	*srb11*Δ::*kanMX4*	SRB/MED, CCR4-NOT	92	23	91
6611	BY4741	*srb2*Δ::*kanMX4*	SRB/MED	76	74	62
4734	BY4741	*srb5*Δ::*kanMX4*	SRB/MED	73	140	**47***
5799	BY4741	*srb8*Δ::*kanMX4*	SRB/MED	95	46	100
5489	BY4741	*med1*Δ::*kanMX4*	SRB/MED	88	120	100
5385	BY4741	*med9*Δ::*kanMX4*	SRB/MED	84	*210*	87
4518	BY4741	*nut1*Δ::*kanMX4*	SRB/MED	100	140	120
4393	BY4741	*pgd1*Δ::*kanMX4*	SRB/MED	58	50	**26**
3119	BY4741	*rox3*Δ::*kanMX4*	SRB/MED	12	*230*	**35***
1976	BY4741	*sin4*Δ::*kanMX4*	SRB/MED, Paf1	85	*500*	100
1742	BY4741	*gal11*Δ::*kanMX4*	SRB/MED, Paf1	24	98	**27***
5326	BY4741	*cdc73*Δ::*kanMX4*	Paf1 complex	94	19	92
4611	BY4741	*rtf1*Δ::*kanMX4*	Paf1 complex	100		
2379	BY4741	*leo1*Δ::*kanMX4*	Paf1 complex	100		
35727	BY4743	*paf1*Δ::*kanMX4*/*paf1*Δ::*kanMX4*	Paf1 complex	50	7.2	26
387	BY4741	*ccr4*Δ::*kanMX4*	CCR4-NOT, Paf1	36	8.5	**4.3**
4072	BY4741	*hpr1*Δ::*kanMX4*	Paf1, THO/TREX	27	5.1	**1.3***
508	BY4741	*mft1*Δ::*kanMX4*	THO/TREX	100		
1191	BY4741	*tex1*Δ::*kanMX4*	THO/TREX	100		
2861	BY4741	*thp2*Δ::*kanMX4*	THO/TREX	100		
4411	BY4741	*dst1*Δ::*kanMX4*	TFIIS	100		
6986	BY4741	*spt4*Δ::*kanMX4*	SPT	100		
LSO2		*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 med2*Δ::*kanMX4*	SRB/MED	36	120	**32***

[a] All strains were from Research Genetics except LSO2 (this study; see Materials and Methods).
[b] All strains contain the markers present in the relevant parental strain.
[c] Resistance to SM was measured as shown in Fig. 1 and quantified as described in the text; values are expressed as percentages of that seen in the corresponding WT parent. Underlined results are <76% of the WT value.
[d] At least three independent transformants of each strain harboring the reporter plasmid pHYC2 were grown under the inducing and noninducing conditions (presence and absence of SM) as described in Materials and Methods, and β-galactosidase activity was assayed in the cell extracts. Mean values were calculated from results that yielded standard deviations of 20% or less and are expressed as percentages of the corresponding values measured in the WT parental strains. Results in italics are 200% of the WT value or greater. Underlined results are <68% of the WT value under inducing conditions and show reductions relative to WT that are 2.5-fold (boldface with an asterisk), 1.5-fold (boldface only), or less than 1.5-fold greater under inducing versus noninducing conditions.
[e] NA, not applicable.
[f] Tfg3p is present in the SWI/SNF, TFIID, NuA3, and TFIIF complexes.

GST pull-down assays were performed (30) as described previously. Samples were resolved on 4 to 12% bis-Tris NuPAGE gels (Invitrogen) with MOPS (morpholinepropanesulfonic acid) buffer according to the manufacturer's protocol. Proteins were transferred to nitrocellulose membranes and detected by immunoblotting. Rabbit polyclonal antisera have been described for Srb7p (47), Swp73p (15), Caf16p (75), and Taf40p (59). Purified rabbit polyclonal Sth1p antibodies were a generous gift from Brad Cairns. Myc-tagged proteins were detected with mouse monoclonal c-Myc (9E10) antibodies from Santa Cruz Biotechnologies (catalog no. sc-40) at a 1:500 dilution.

Yeast cultures for Western analysis of HA_3-Gcn4p were grown and induced as described above for the reporter gene assays. Extracts were prepared as described previously (29) except that additional protease inhibitors were added (Roche complete inhibitor used at a 1× concentration), and the cells were lysed in 14-ml tubes by using 10 cycles of 30 s of vortexing with 1.5 min of cooling on ice between cycles. Protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce). Proteins were resolved on 10% bis-Tris NuPAGE gels (Invitrogen) in MOPS buffer at a constant voltage of 200 V for 1.5 h with the gel chamber submerged in ice. After transfer of the proteins to nitrocellulose, the membranes were cut and probed with rabbit polyclonal HA antibodies from Santa Cruz Biotechnologies (probe Y-11; catalog no. sc-805) at a dilution of 1:500 to detect HA_3-Gcn4p and with Gcd6p antibodies (14).

Cultures were grown and induced for Northern analysis as described above or in some cases by using the inducing conditions described below for ChIP assays. Total RNA was extracted and subjected to Northern analysis as described previously (92). Probes were prepared from PCR amplified yeast genomic DNA or as restriction fragments from plasmid clones by random prime labeling using the RediPrime II system (Invitrogen).

Chromatin immunoprecipitations. Yeast cells were grown as described above for reporter assays except that SM induction was carried out for 2 h. Living cells were fixed with 1% formaldehyde and broken by vortexing as described previously (62). Lysates were collected and sonicated to produce chromatin fragments of 200 to 1,000 bp, with an average size of ~500 bp. After sonication, the chromatin extracts were clarified by centrifugation for 1 h. Chromatin immunoprecipitation was conducted as described (24). Quantitative PCRs contained 1× Platinum *Taq* polymerase buffer (Invitrogen), 1.5 mM Mg_2Cl_2, 0.2 mM deoxynucleoside triphosphate, 1.6 μCi of [^{33}P]dATP (Amersham), 0.5 μM *POL1* primer pair, 0.15 μM *ARG1$_{UAS}$* primer pair, 1.5 U of Platinum *Taq* polymerase (Invitrogen), and 1/10 of the immunoprecipitated chromatin sample or 1,000-fold diluted input DNA in 15-μl reaction volumes. PCR parameters were 94°C for 4 min; 94°C for 30 s, 52°C for 30 s, and 65°C for 1 min for 26 cycles; and then 65°C for 5 min. PCR products were resolved on 6% polyacrylamide gels and quantified by phosphorimaging analysis.

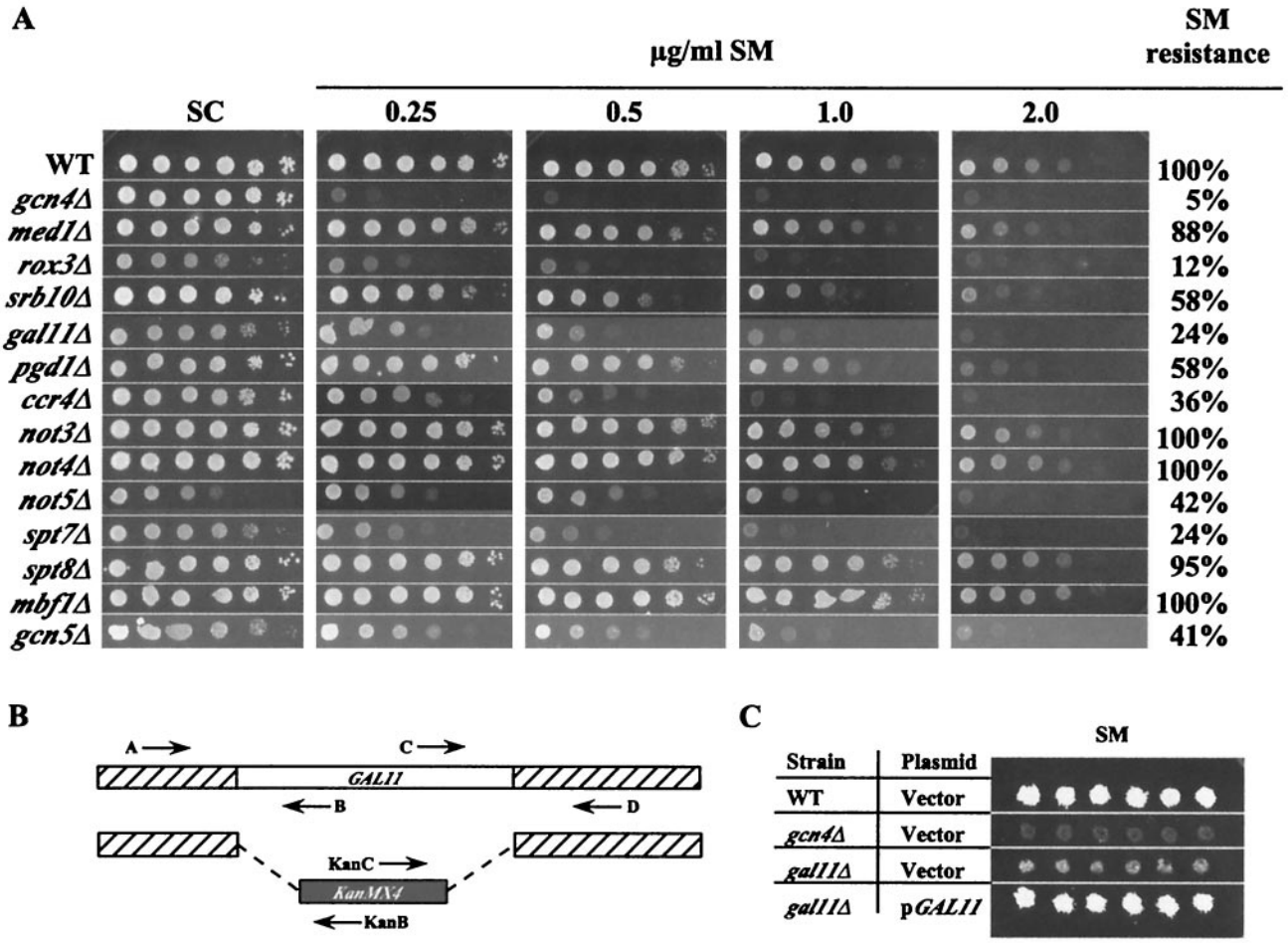


FIG. 1. Analysis of SM resistance and physical and genetic assays used to confirm the identity of deletion strains. (A) Tenfold serial dilutions of the isogenic WT, *gcn4*Δ, and relevant deletion strains grown in SC medium were spotted to SC (control) medium or SC−ILV medium containing SM at 0.25, 0.5, 1.0, and 2.0 μg/ml. Growth on each plate after 3 days at 25°C was scored and adjusted for slow growth on SC medium, and these values were used to generate semiquantitative scores expressed as a percentage of the WT score on the same plate (SM resistance). (B) Schematic representation of PCR confirmation of deletions. For each gene of interest an upstream primer (A) or downstream primer (D) was used in conjunction with internal primers (B or C, respectively) to identify the WT allele or with primers specific for the *kanMX4* sequences (primer KanB or KanC, respectively) to identify the deletion. All primer sequences were the same as those used by the *Saccharomyces* Genome Deletion Project for confirmation of the deletions (listed at http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). (C) Example of the complementation assay used to confirm the identity of mutants displaying SM resistance less than 85% of that of the WT. A plasmid containing the WT gene corresponding to the relevant deletion (in this case *GAL11*; see Table 2 for a complete list of plasmids), or the empty vector, was introduced into the mutant strain, and transformants were tested by replica plating for sensitivity to SM.

The sequences of the *POL1* primer pair are 5′ GAC AAA ATG AAG AAA ATG CTG ATG CAC C 3′ (positions 2477 to 2504, with the A of the start codon as position 1) and 5′ TAA TAA CCT TGG TAA AAC ACC CTG 3′ (positions 2730 to 2707). The sequences of the $ARG1_{UAS}$ primer pair are 5′ ACG GCT CTC CAG TCA TTT AT 3′ (−378 to −359, with the A of the start codon as +1) and 5′ GCA GTC ATC AAT CTG ATC CA 3′ (−213 to −232).

RESULTS

A comprehensive analysis of nonessential coactivator subunits reveals novel requirements for activation by Gcn4p. Mutants from the *Saccharomyces* Genome Deletion Project (136) harboring deletions in each of 80 genes encoding proteins implicated as coactivators or corepressors (Table 1) were tested for sensitivity to SM, an inhibitor of leucine, isoleucine, and valine biosynthesis (65). Mutants with defects in Gcn4p-mediated induction of enzymes in these pathways are unable to grow on SM-containing medium (Gcn^- phenotype) (55, 93). Tenfold serial dilutions of each strain were spotted on SC−ILV medium and containing various concentrations of SM. Each strain was assigned a numerical score based on its degree of growth at each dilution for four different concentrations of SM, and the resulting scores were expressed as a percentage of that assigned to the WT. This analysis provided a semiquantitative assessment of SM resistance for each strain (SM^r) (Fig. 1A). We also analyzed the growth of the mutants on SC medium without SM to identify mutants with slow-growth (Slg^-) phenotypes, and we adjusted the SM^r scores accordingly (e.g., *rox3*Δ and *not5*Δ in Fig. 1A). The resulting SM^r phenotypes of all of the strains are listed in Table 1 and plotted in the histograms shown in Fig. 2A and B and Fig. 3B and C. For

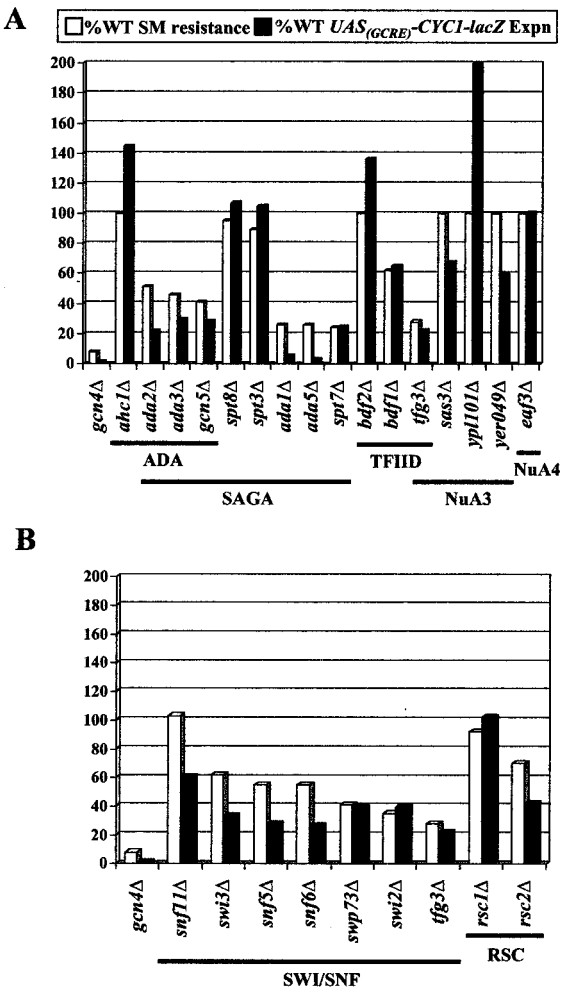
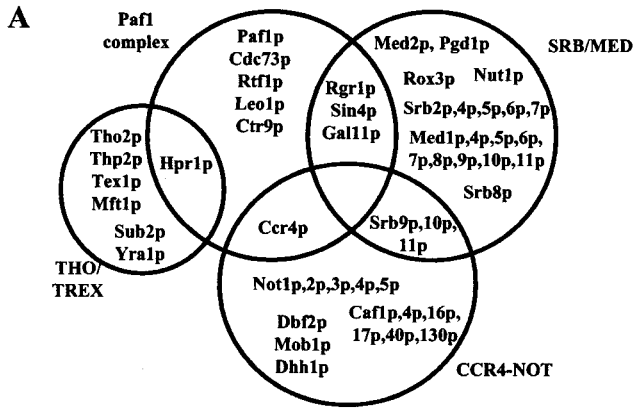


FIG. 2. Phenotypes of deletion mutants lacking subunits of chromatin-modifying complexes. The SM resistance measured as described for Fig. 1A and induced UAS_{GCRE}-*CYC1-lacZ* expression measured as described in Table 1 for each deletion strain are shown graphically as percentages of the WT values. The values for the UAS_{GCRE}-*CYC1-lacZ* reporter are averages for three independent transformants induced with SM. Standard deviations, all less than 20% of the mean values, have been omitted for clarity. (A) HAT-containing complexes; (B) chromatin-remodeling complexes.

every mutant judged to have a level of SM resistance that was <85% of that of the WT, we confirmed that its SM-sensitive phenotype (SM^s) was complemented by a plasmid-borne copy of the WT gene (Table 2; e.g., *gal11*Δ in Fig. 1C). PCR analysis of genomic DNA was carried out to verify the presence of the relevant deletion (Fig. 1B) in the remaining mutants with SM resistance that was >85% of that of the WT.

Because Gcn4p target genes are often regulated by more than one transcriptional activator (48), the SM^s phenotype could arise from defective activation of an isoleucine-valine biosynthetic (*ILV*) gene by an activator besides Gcn4p. To measure activation by Gcn4p more directly, we assayed expression of a Gcn4p-dependent *lacZ* reporter in cells treated with SM, in which Gcn4p synthesis is induced at the translational level (134). This UAS_{GCRE}-*CYC1-lacZ* reporter contains two copies of a Gcn4p binding site from *HIS4* (UAS_{GCRE}) inserted at the *CYC1* promoter in place of the endogenous upstream activation sequence (UAS) element. It is induced by a factor of 20 to 30 in WT cells (typically, from 75 to 2,000 U of β-galactosidase activity) and is almost completely dependent on Gcn4p (50), giving only 1% of WT expression in the *gcn4*Δ mutant (Table 1 and Fig. 2A). At least three independent transformants of each SM^s strain harboring the reporter plasmid were assayed for β-galactosidase activity under inducing and noninducing conditions (i.e., presence and absence of SM), and the mean values are listed in Table 1 and graphed in Fig. 2 and 3. In all cases, the standard deviations were less than 20% of the means.

With only one exception (*dbf2*Δ), all of the mutants whose resistance to SM was less than 75% of that of the WT also were impaired for induction of the UAS_{GCRE}-*CYC1-lacZ* reporter, showing less than 65% of WT expression under inducing conditions. The results for the 27 mutants fulfilling these criteria are presented in Table 1 and in Fig. 2 and 3. Although the



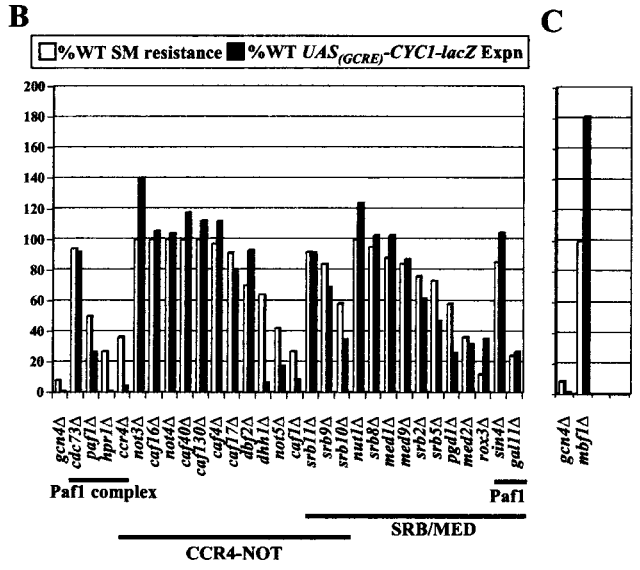


FIG. 3. Phenotypes of mediator and related complexes and miscellaneous mutants. (A) Venn diagram depicting the relationships among the subunits of CCR4-NOT, SRB/MED, the Paf1 complex, and THO/TREX; (B) graphic representation of the phenotypes of deletion mutants lacking subunits of these complexes, as shown in Fig. 2; (C) phenotypes of the *mbf1*Δ strain analyzed as for panel B.

TABLE 2. Plasmids used for complementation assay

Gene	Plasmid	Source
ADA1	YCp50-ADA1	52
ADA2	pNS3.8	10
ADA3	pADA3-HHV	100
ADA5	pSR36	108
BDF1	BDF1/pRS316	B. Futcher
CAF1	YCp33/CAF1	This study
CCR4	YEp213 + CCR4	46
DHH1	YEp213 + DHH1	46
GAL11	pJF111	37
GCN5	GCN5/pRS316	81
HPR1	pHK229	H. Klein
MED2	pGM26	87
NOT5	pRS316/NOT5	This study
PAF1	YCplac33/PAF1	This study
PGD1	p316HRS1-11	111
ROX3	YCp(33)ROX3H	36
RSC2	316.RSC2	16
SPT7	pFW127	F. Winston
SNF5	pAC153	1
SNF6	pEL3.10	35
SRB2	pCT24	R. Young
SRB5	pCT39	128
SRB10	pRM57	This study
SW12	pLN138-4	1
SW13	pRS316/SW13	This study
SWP73	pUCA-SWP73	15
TFG3	316.TFG3	B. Cairns

*dbf2*Δ mutant was not significantly impaired for UAS_{GCRE}-*CYC1-lacZ* expression, it was defective for induction of a *HIS3-GUS* reporter described below. In general, the SM^s phenotype correlated reasonably well with the quantitative defects in UAS_{GCRE}-*CYC1-lacZ* induction (Fig. 2A and B and 3B). In the *hpr1*Δ, *ccr4*Δ, and *dhh1*Δ strains, however, reporter induction was considerably more impaired than was expected from the severity of SM sensitivity (Fig. 3B and Table 1). This discrepancy could be explained in several ways. One possibility is that other activators responsible for basal expression of the *ILV* genes are less dependent on Hpr1p, Ccr4p, or Dhh1p function than is Gcn4p. Alternatively, the mutations could have a greater effect on the function of the *CYC1* core promoter in the UAS_{GCRE}-*CYC1-lacZ* construct than on the core promoters of *ILV* genes. Finally, it was shown previously that *hpr1* mutations impede transcriptional elongation through *lacZ* sequences (18), and this could lead to a greater impairment of UAS_{GCRE}-*CYC1-lacZ* expression than with authentic Gcn4p target genes.

Expression of UAS_{GCRE}-*CYC1-lacZ* was reduced in the *hpr1*Δ strain by a factor of 77 under inducing conditions but by a factor of only 20 under noninducing conditions (Table 1). Thus, while the low level of UAS_{GCRE}-*CYC1-lacZ* expression in the *hpr1*Δ mutant probably reflects an elongation defect, the fact that this mutation had a greater effect on reporter expression under inducing than noninducing conditions suggests that transcriptional activation by Gcn4p is also impaired. If the effect of a coactivator mutation on UAS_{GCRE}-*CYC1-lacZ* expression was greater under inducing than noninducing conditions by a factor of 2.5 or more, we concluded that transcriptional activation was damaged in that strain, in addition to other defects in elongation or core promoter function that might exist. The group of mutants that satisfied this criterion is identified in Table 1. In addition to *hpr1*Δ, this group also includes *not5*Δ, *caf1*Δ (affecting CCR4/NOT), and multiple mutations in subunits of SAGA, SWI/SNF, and SRB/MED.

A second group of mutants that did not satisfy this rigorous criterion but were comparable to *gcn5*Δ in their relative impairment of reporter expression under inducing and noninducing conditions are also shown in Table 1. Mutants in this second group include *ada1*Δ (SAGA), *ccr4*Δ and *dhh1*Δ (affecting the Paf1 complex or CCR4/NOT), *pgd1*Δ (SRB/MED), *snf11*Δ (SWI/SNF), *yer049*Δ (NuA3), and *tfg3*Δ (affecting multiple complexes) strains. Given the considerable evidence that Gcn5p is a bone fide coactivator for Gcn4p (63, 64, 126), it seems likely that many mutants in this second class also are defective for transcriptional activation by Gcn4p. A third group of five mutants in Table 1 reduced UAS_{GCRE}-*CYC1-lacZ* expression to the same, or even greater, extent under noninducing versus inducing conditions, making it unclear whether activation by Gcn4p was defective in these strains.

Finally, there were mutations affecting subunits of SAGA (*spt3*Δ and *spt8*Δ), NuA3 (*ypl101*Δ), SWI/SNF (*swi2*Δ), CCR4/NOT (*caf17*Δ), and SRB/MED (*med9*Δ, *rox3*Δ, and *sin4*Δ) that led to expression levels two- to fivefold higher than WT levels under noninducing conditions (Table 1). Such mutations may eliminate a negative regulatory mechanism that represses *CYC1* promoter activity at low levels of Gcn4p. Interestingly, the *swi2*Δ and *rox3*Δ mutations reduced UAS_{GCRE}-*CYC1-lacZ* expression under inducing conditions, indicating dual positive and negative functions at this promoter for these subunits of SWI/SNF and SRB/MED (Table 1).

The array of coactivator subunits required for activation by Gcn4p varies at different promoters. In an effort to confirm the conclusions reached above, we assayed a subset of the mutants for expression of a second Gcn4p-dependent reporter containing the 5′ noncoding region of *HIS3* from position −450 to −3 (relative to the ATG start codon) fused to *GUS* coding sequences. As above, at least three independent transformants of each strain harboring the reporter plasmid were assayed for β-glucuronidase activity under inducing and noninducing conditions, and the mean values are listed in Table 3. In all cases, the standard errors were less than 20% of the mean values. The *HIS3-GUS* reporter is induced three- to eightfold by SM treatment of WT cells (data not shown), and ~90% of its expression under these conditions is dependent on Gcn4p (Table 3). The residual *HIS3-GUS* expression in the *gcn4*Δ mutant can be attributed to the AT-rich element that confers Gcn4p-independent promoter activity (122). Because *HIS3-GUS* expression is ~5-fold more dependent on Gcn4p under inducing than noninducing conditions (Table 3), by comparing the relative impairment of reporter expression under these conditions, we could evaluate whether the coactivator mutants are defective for activation of *HIS3-GUS* by Gcn4p. The data are coded according to criteria similar to those employed above for the UAS_{GCRE}-*CYC1-lacZ* reporter (see the legend to Table 3 for details). The results suggest that Hpr1p, the Cdc73p subunit of the Paf1 complex, RSC subunit Rsc1p, and multiple subunits of SAGA, SRB/MED, and CCR4/NOT are all required for full activation of the *HIS3* promoter by Gcn4p (Table 3).

Interestingly, the *spt3*Δ mutant was impaired for *HIS3-GUS* induction (Table 3), even though it showed WT induction of

TABLE 3. *HIS3-GUS* expression phenotypes of cofactor mutants

Relevant genotype	Cofactor complex	β-Glucuronidase activity (% of WT) for[a]:	
		Uninduced *HIS3-GUS*	Induced *HIS3-GUS*
*gcn4*Δ	Activator	55	11
*mbf1*Δ	NA[b]	76	**27***
*ada2*Δ	ADA, SAGA	86	**16***
*ada3*Δ	ADA, SAGA	143	**20***
*gcn5*Δ	ADA, SAGA	29	**6.4***
*spt8*Δ	SAGA	*289*	84
*spt3*Δ	SAGA	141	**29***
*ada1*Δ	SAGA	149	**20***
*ada5*Δ	SAGA	*235*	**32***
*spt7*Δ	SAGA	46	**9.1***
*tfg3*Δ	Multiple[c]	113	**58**
*rsc1*Δ	RSC	15	**4.2***
*rsc2*Δ	RSC	23	49
*swi2*Δ	SWI/SNF	*523*	169
*swi3*Δ	SWI/SNF	43	**25**
*snf5*Δ	SWI/SNF	*363*	117
*snf6*Δ	SWI/SNF	147	82
*snf11*Δ	SWI/SNF	112	83
*swp73*Δ	SWI/SNF	*437*	129
*cdc73*Δ	Paf1 complex	117	**42***
*hpr1*Δ	Paf1 complex, THO/TREX	13	**3.3***
*ccr4*Δ	CCR4-NOT, Paf1 complex	25	**10***
*not3*Δ	CCR4-NOT	58	52
*caf16*Δ	CCR4-NOT	76	**40**
*not4*Δ	CCR4-NOT	67	51
*caf40*Δ	CCR4-NOT	58	**33**
*caf130*Δ	CCR4-NOT	56	46
*caf4*Δ	CCR4-NOT	86	**54**
*caf17*Δ	CCR4-NOT	97	**64**
*dbf2*Δ	CCR4-NOT	40	**20***
*dhh1*Δ	CCR4-NOT	16	12
*not5*Δ	CCR4-NOT	67	**39**
*caf1*Δ	CCR4-NOT	53	**10***
*srb11*Δ	SRB/MED, CCR4-NOT	140	77
*srb9*Δ	SRB/MED, CCR4-NOT	53	**12***
*srb10*Δ	SRB/MED, CCR4-NOT	*227*	73
*pgd1*Δ	SRB/MED	*469*	*259*
*med2*Δ	SRB/MED	97	**43***
*rox3*Δ	SRB/MED	92	**21***
*sin4*Δ	SRB/MED, Paf1 complex	*253*	119
*ga111*Δ	SRB/MED, Paf1 complex	145	**55***

[a] At least three independent transformants of each strain harboring the reporter plasmid pKN7 were grown under inducing and noninducing conditions (presence and absence of SM) as described in Materials and Methods, and β-glucuronidase activity was assayed in the cell extracts. Mean values were calculated from results that yielded standard deviations of 20% or less and expressed as percentages of the corresponding values measured in the WT parental strains. Results in italics are 200% of the WT value or greater. Underlined results are <68% of the WT value under inducing conditions and show reductions relative to the WT that are 2-fold (boldface with an asterisk), 1.5-fold (boldface only), or less than 1.5-fold greater under inducing than noninducing conditions.

[b] NA, not applicable.

[c] Tfg3p is present in the SWI/SNF, TFIID, NuA3, and TFIIF complexes.

UAS_{GCRE}-CYC1-lacZ (Table 1). Hence, the requirement for this SAGA subunit in activation by Gcn4p seems to vary between these two promoters. This last phenomenon also holds for the CCR4/NOT subunit Dbf2p, the SRB/MED subunit Srb9p, Rsc1p, and Mbf1p. The SRB/MED subunit Pgd1p showed the opposite behavior, as *pgd1*Δ cells were strongly impaired for *UAS_{GCRE}-CYC1-lacZ* induction (Table 1) but showed greater-than-WT induction of *HIS3-GUS*. Thus, Pgd1p may have a role in negative control that overrides its importance as a coactivator for Gcn4p at *HIS3-GUS*. This conclusion is consistent with previous results indicating dual positive and negative functions for Pgd1p at other promoters (101). Whereas the *rsc1*Δ mutation had a greater effect than *rsc2*Δ on *HIS3-GUS* expression (Table 3), the opposite was true for the *UAS_{GCRE}-CYC1-lacZ* reporter (Table 1). There are two forms of RSC that contain either Rsc1p or Rsc2p (16). Thus, it seems that Gcn4p utilizes both forms of RSC, but the relative importance of the Rsc1p- and Rsc2p-containing complexes depends on the promoter.

It was also important to analyze the effects of coactivator mutations on induction of authentic mRNAs by Gcn4p. Accordingly, we prepared total RNA from mutant and WT strains and conducted Northern analysis using probes specific for the Gcn4p target genes *SNZ1*, *HIS4*, *ARG1*, and *ILV2* (94) and for *ACT1*, analyzed as an internal control. RNA was prepared from two independent cultures of each strain, and the Northern signals for the Gcn4p-regulated mRNAs were quantified and normalized to the *ACT1* signals. Typical Northern data are presented in Fig. 4 for the duplicate determinations of selected mutants to illustrate the reproducibility of these data. The mean normalized mRNA levels for the entire set of mutants are given in Table 4.

SNZ1 mRNA is an interesting case because it resembles the *UAS_{GCRE}-CYC1-lacZ* construct in showing very low levels of expression in noninducing conditions and a strong dependence on Gcn4p under inducing conditions (94). As shown in Fig. 4A and Table 4, mutations in multiple subunits of SAGA, SWI/SNF, and SRB/MED, Rsc1p (RSC), Dhh1p (CCR4/NOT), Ccr4p (CCR4/NOT and Paf1), Hpr1p (Paf1 and THO/TREX), and Mbf1p all lowered the induced level of *SNZ1* mRNA to below 68% of WT levels. Surprisingly, deletion of *GCN5* led to slightly greater than WT levels of *SNZ1* mRNA. Although WT transcriptional induction of *SNZ1* by Gcn4p requires the same coactivator complexes needed for high-level expression of the reporter constructs, the subunits most critically required differ somewhat among the *SNZ1*, *HIS3-GUS*, and *UAS_{GCRE}-CYC1-lacZ* promoters.

Efficient induction of *HIS4* mRNA was dependent on multiple subunits of SAGA and SRB/MED and also required Rsc2p, Swp73p (SWI/SNF), Ccr4p, and Hpr1p (Paf1p and THO/TREX) (Fig. 4B and Table 4). Surprisingly, deleting the ATPase subunit of SWI/SNF (Swi2p) led to greater-than-WT *HIS4* expression, whereas inactivation of the Swp73p subunit significantly reduced *HIS4* induction. It was shown recently that Swi2p can function as a repressor independently of other SWI/SNF subunits (82). Thus, perhaps Swi2p acts in this manner as a repressor at *HIS4* and also in conjunction with other SWI/SNF subunits in activation and the former activity has the greater impact on *HIS4* transcription. Again, the array of subunits of SAGA, SRB/MED, CCR4/NOT, SWI/SNF, and RSC critical for activation by Gcn4p differs somewhat between *SNZ1* and *HIS4*: Swi2p, Rsc1p, Dhh1p, Med2p, Sin4p, and Gal11p are required at *SNZ1* but not at *HIS4*, whereas Ada2p and Rsc2p are more important at *HIS4* than at *SNZ1*.

ILV2 mRNA induction was substantially impaired by mutations in several subunits of SAGA and CCR4/NOT and in *MBF1* and by deletion of *ROX3* (SRB/MED) (Table 4). Thus, with the possible exception of SWI/SNF, high-level activation of *ILV2* requires at least one subunit of the same coactivator

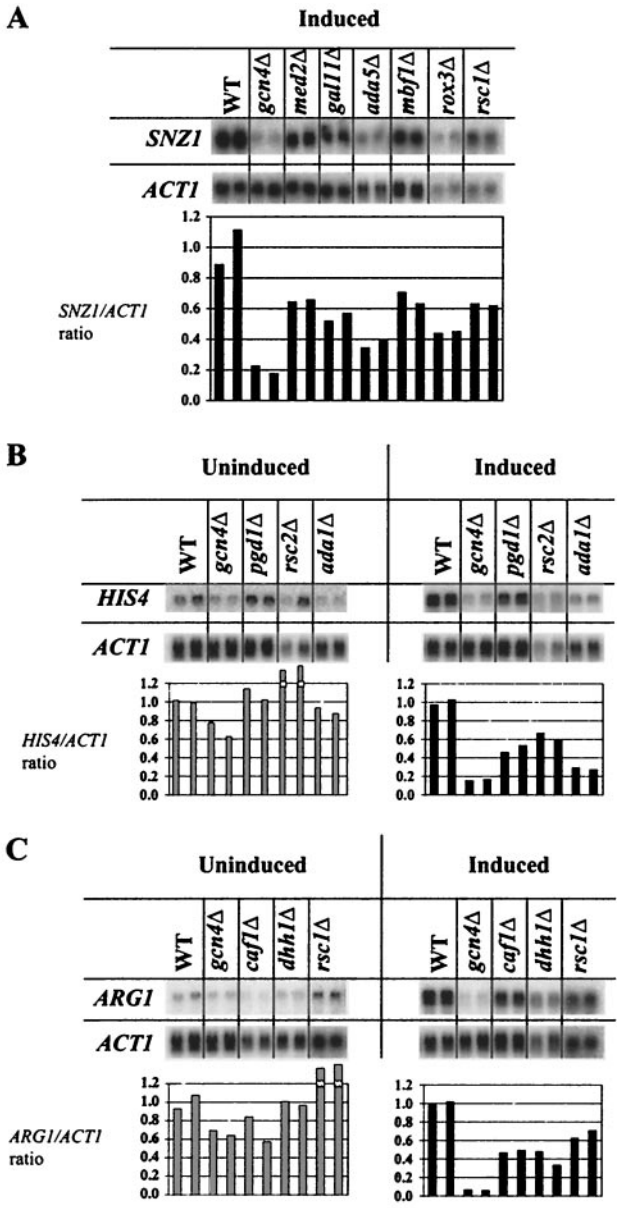


FIG. 4. Northern analysis of authentic Gcn4p target genes in a typical subset of deletion mutants. Total RNA was isolated for each strain under the inducing and noninducing conditions described in Table 1, and equal amounts of RNA were subjected to Northern analysis, probing for *ACT1*, *ARG1*, *HIS4*, *ILV2*, and *SNZ1* mRNAs. Adjacent lanes contain RNA samples isolated from two independent cultures for each strain. The hybridization signals were quantified with a PhosphorImager (Molecular Dynamics, with ImageQuant 5.2 software), and the values obtained for *SNZ1* (A), *HIS4* (B), and *ARG1* (C) were normalized to the corresponding *ACT1* signals. The resulting ratios calculated for the mutant strains were normalized to the ratio measured in the WT, and the normalized ratios are plotted in histograms beneath the corresponding lanes of each blot.

complexes needed for activation of other Gcn4p-dependent promoters. We noted that a number of mutants with a SM^s phenotype displayed WT or greater levels of *ILV2* mRNA (Table 4). Presumably, these mutants are defective for induction of one of the other four *ILV* genes rather than *ILV2* (58).

The coactivator requirements for activation of *ARG1* transcription are unique. As for certain other Gcn4p target genes, high-level induction was impaired by mutations in multiple subunits of CCR4/NOT and SRB/MED, Rsc1p, and Hpr1p (Table 4 and Fig. 4C). However, *ARG1* induction showed no significant dependence on any SAGA subunit. Moreover, nearly all of the SAGA mutations led to higher *ARG1* expression under noninducing conditions (Table 4). The latter data are consistent with the finding that SAGA is required for arginine-specific repression of *ARG1* by the ArgR/Mcm1p repressor complex (107). The fact that SAGA mutations did not produce higher-than-WT *ARG1* expression under inducing conditions (Table 4) may indicate that activation of this promoter by Gcn4p is SAGA dependent, as observed for all other Gcn4p target genes. In this view, the SAGA subunit deletions have offsetting positive and negative effects on transcription by simultaneously impairing repression by ArgR/Mcm1p and activation by Gcn4p. Considering the other coactivator mutations that derepressed *ARG1* mRNA under noninducing conditions (Table 4), we suggest that RSC, SWI/SNF, SRB/MED, and the Paf1 complex may also be required for *ARG1* repression. The derepressing effect of the *paf1*Δ mutation on *ARG1* mRNA is particularly striking.

Coactivator mutants with SM^s phenotypes generally do not have reduced levels of Gcn4p. A mutation may impair the activation of Gcn4p target genes by lowering the induced level of Gcn4p. To distinguish such mutants from those truly defective in activation, we measured the steady-state levels of an HA epitope-tagged version of Gcn4p by Western analysis in each SM^s mutant. The relevant strains were transformed with a single-copy plasmid expressing HA_3-Gcn4p from the native promoter (*CEN*/*GCN4-HA*$_3$), and WCEs were prepared following growth in the presence of SM to induce HA_3-Gcn4p expression. Western analysis using anti-HA antibodies showed that, as expected, HA_3-Gcn4p levels increased dramatically on treatment of WT transformants with SM (data not shown). In 20 of 27 mutants tested, the levels of HA_3-Gcn4p were greater than or equal to those in the WT strain. An example of the data obtained for one such mutant (*ccr4*Δ) is shown in Fig. 5A (see Table 5 for results on all mutants).

In the remaining seven mutants, we saw a significant reduction in the levels of HA_3-Gcn4p relative to the WT strain (Table 5), ranging from 20% (*dhh1*Δ) to 40% (*ada1*Δ, *ada5*Δ/*spt20*Δ, *bdf1*Δ, *hpr1*Δ, *not5*Δ, and *swi3*Δ) of the WT level. To determine whether the SM^s phenotypes of this class resulted from low levels of the activator, we increased expression of HA_3-Gcn4p by introducing *GCN4-HA*$_3$ on a high-copy-number plasmid (2μm/*GCN4-HA*$_3$). In five of these mutants (*ada1*Δ, *ada5*Δ/*spt20*Δ, *dhh1*Δ, *hpr1*Δ, and *not5*Δ), the levels of HA_3-Gcn4p equaled or exceeded that in the WT strain containing single-copy *GCN4-HA*$_3$, and the SM sensitivity remained unchanged. An example of this behavior is illustrated in Fig. 5B and C for the *not5*Δ mutant. We conclude that the SM^s phenotypes of these five strains cannot be accounted for by reduced levels of Gcn4p and that Gcn4p activation function is impaired. It is also noteworthy that *hpr1*Δ cells showed no activation defect at *ILV2*, that *dhh1*Δ did not impair *HIS4* induction, and that *not5*Δ cells showed normal induction of *SNZ1* mRNA (Table 4). Thus, the reductions in Gcn4p levels observed in these three mutants are not great enough to impair the activation of all Gcn4p-dependent promoters. Transfor-

TABLE 4. Northern blot analysis of cofactor mutants

Relevant genotype	Cofactor complex	Mean normalized signal ratio (% of WT)[a]						
		SNZ1, induced	*HIS4*		*ILV2*		*ARG1*	
			Uninduced	Induced	Uninduced	Induced	Uninduced	Induced
*gcn4*Δ	Activator	12[b,c]	67[b,c]	31[b,c]	78[c]	32[c]	51[b,c]	14[b,c]
*mbf1*Δ	NA[d]	63[c]		73[c]		68[c]		77[c]
*ada2*Δ	ADA, SAGA	70[b]	47[b]	43[b]			*207*[b]	96[b]
*gcn5*Δ	ADA, SAGA	124[b,c]	105[b,c]	98[b,c]	38[c]	71[c]	*313*[b,c]	143[b,c]
*ada1*Δ	SAGA	47[c]	90[c]	**29***[c]	69[c]	59[c]	*915*[c]	101[c]
*ada5*Δ	SAGA	45[c]		27[c]		45[c]		106[c]
*spt3*Δ	SAGA	68[b]	69[b]	52[b]			130[b]	86[b]
*spt7*Δ	SAGA	41[b]					*458*[b]	121[b]
*swi2*Δ	SWI/SNF	44[b,c]	139[b]	*206*[b]		109[c]		118[b,c]
*swp73*Δ	SWI/SNF	30[b]		32[b]				
*rsc1*Δ	RSC	67[c]	118[c]	88[c]	88[c]	70[b,c]	198[c]	**67***[c]
*rsc2*Δ	RSC	144[b,c]	*242*[c]	**63**[c]	99[c]	124[c]	*>600*[c]	111[c]
*not5*Δ	CCR4-NOT	155[c]	118[c]	74[c]	85[c]	79[c]	91[c]	72[c]
*caf1*Δ	CCR4-NOT	76[b,c]	103[c]	77[c]	51[c]	62[c]	71[c]	**48**[c]
*dhh1*Δ	CCR4-NOT	65[b,c]	135[b,c]	102[b,c]	96[c]	**56***[c]	99[c]	**41***[c]
*srb5*Δ	SRB/MED	126[b,c]	*307*[c]	118[c]	152[c]	92[c]	*>300*[b,c]	148[b,c]
*pgd1*Δ	SRB/MED	62[c]	144[b,c]	**67***[b,c]	114[c]	72[c]	*474*[c]	**57***[c]
*rox3*Δ	SRB/MED	38[b,c]	129[b,c]	**67***[b,c]	83[c]	62[c]	105[b,c]	**52***[b,c]
*sin4*Δ	SRB/MED, Paf1	68[c]	125[c]	94[c]	91[c]	86[c]	*379*[c]	85[c]
*gal11*Δ	SRB/MED, Paf1	55[c]	78[b,c]	102[b,c]	118[c]	78[c]	*213*[b,c]	97[b,c]
*med2*Δ	SRB/MED	65[c]	76[c]	84[c]	168[c]	88[c]	171[c]	83[c]
*cdc73*Δ	Paf1 complex	98[c]		84[c]		77[c]		100[c]
*ccr4*Δ	CCR4-NOT, Paf1	57[b,c]	70[b,c]	49[b,c]	60[c]	51[c]	70[c]	**44**[c]
*hpr1*Δ	Paf1, THO/TREX	49[b,c]	59[b,c]	50[b,c]	54[c]	125[c]	65[c]	66[c]
*gcn4*Δ/*gcn4*Δ	Activator	16[c]	57[c]	23[c]	81[c]	26[c]	54[c]	7[c]
*paf1*Δ/*paf1*Δ	Paf1 complex	97[c]	*327*[c]	126[c]	120[c]	136[c]	*2,770*[c]	149[c]
*snf5*Δ/*snf5*Δ	SWI/SNF	75[c]	93[c]	85[c]	*261*[c]	162[c]	*500*[c]	104[c]

[a] For each value shown, at least two independent cultures of each strain were grown under inducing or noninducing conditions (presence or absence of SM) as described in Materials and Methods, and equal amounts of isolated total RNA were subjected to Northern analysis. The hybridization signals for *SNZ1*, *HIS4*, *ILV2*, and *ARG1* were normalized to corresponding *ACT1* signals. The resulting ratios calculated for the mutant strains were normalized to the ratio measured for the WT, and the mean normalized ratios are shown as a percentage of the WT value. The mean values were calculated from results that yielded standard errors of 30% or less. Results in italics are 200% of the WT value or greater. Underlined results are <68% of the WT value under inducing conditions and show reductions relative to WT that are ~2-fold (bold face type with an asterisk), 1.5-fold (bold face type), or less than 1.5-fold greater under inducing than noninducing conditions.

[b] Induction as per the ChIP assay.

[c] Induction as per the reporter assay.

[d] NA, not applicable.

mants of the *bdf1*Δ and *swi3*Δ mutants harboring the 2μm/*GCN4-HA*$_3$ plasmid had levels of HA$_3$-Gcn4p exceeding that of the WT strain bearing *CEN*/*GCN4-HA*$_3$; however, their SM^s phenotypes were partially complemented (Table 5). Thus, the activation defects in the *bdf1*Δ and *swi3*Δ strains may result partly from reduced Gcn4p expression.

Mutations in enzymes affecting chromatin structure generally do not disrupt activation by Gcn4p. Since Gcn4p requires a number of complexes involved in chromatin modification, it was possible that any perturbation of chromatin structure might affect activation by Gcn4p. To address this possibility, we examined mutants with deletions of other nonessential HATs, histone deacetylases and ATP-dependent chromatin-remodeling enzymes for growth in the presence of SM. Among the known HAT mutants, only the *gcn5*Δ strain showed sensitivity to SM (Fig. 6A). Interestingly, deletion of the HAT subunit of SRB/MED, Nut1p, or the transcription Elongator complex, Elp1p, had no effect on SM resistance, as did deletions of two other members of Elongator, Elp2p and Elp3p (Fig. 6A and data not shown). To address the possibility of redundant contributions of different HATs to activation by Gcn4p, we generated double deletions of various HATs with *gcn5*Δ or *nut1*Δ and assayed growth on SM. Double mutants containing a deletion of *GCN5* displayed phenotypes nearly identical to that of the *gcn5*Δ single mutant (Fig. 6B and data not shown). The *gcn5*Δ *sas3*Δ strain was not viable, consistent with previous findings (53). Combining deletions of various HATs with the *nut1*Δ mutation also did not reveal any additive SM^s phenotypes (Fig. 6B and data not shown). Although histone deacetylases are normally associated with transcriptional repression, some deacetylase mutants show decreased transcription of specific genes (11); however, we detected no SM sensitivity for any deacetylase mutant (Table 1).

As Gcn4p requires both SWI/SNF and the highly related RSC complex for WT activation (Fig. 2B), we assayed deletions of the nonessential subunits of other chromatin-remodeling complexes. As shown in Fig. 6C, we observed no SM sensitivity in mutants lacking nonessential subunits of the ISWI complexes (*isw1*Δ, *isw2*Δ, and *itc1*Δ) and Chd1p, which functions as a homodimer and can affect transcription both positively and negatively (129). Thus, the SM^s phenotypes of the SWI/SNF and RSC mutants reflect a specific requirement for these complexes in activation by Gcn4p rather than a general perturbation of chromatin structure.

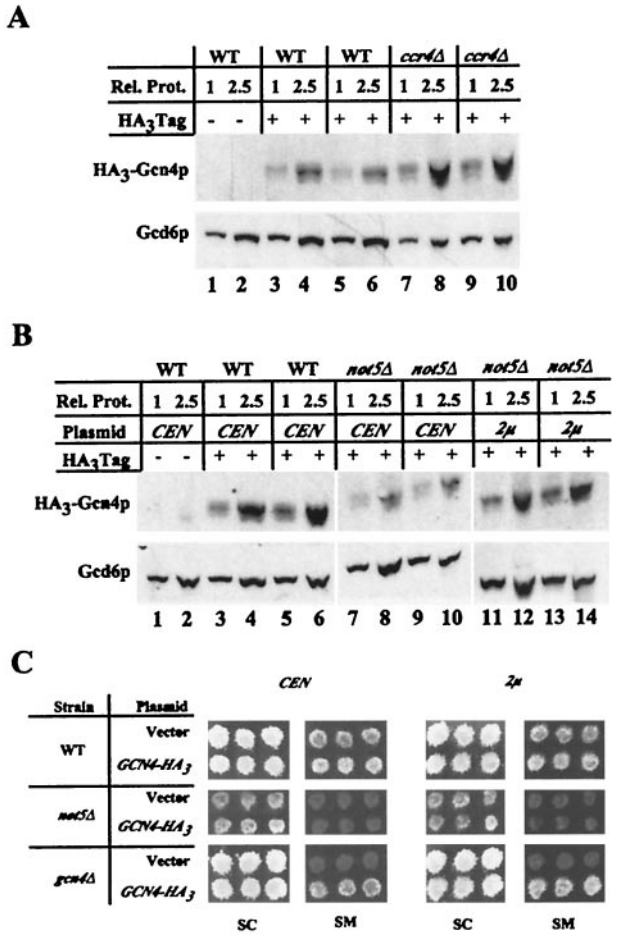


FIG. 5. Western analysis of Gcn4p levels in Gcn$^-$ mutants. Single-copy plasmid p2382 (*CEN*) or high-copy-number plasmid pHQ1239 (2μm) harboring the *GCN4-HA$_3$* allele was introduced into the WT and Gcn$^-$ deletion strains, and the WT strain was also transformed with empty *CEN* or 2μm vector. (A and B) Extracts were prepared from two transformants of each strain induced with SM. Two amounts (20 and 50 μg) of total protein, labeled as relative protein (Rel. Prot.) amounts of 1 and 2.5, respectively, were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with Gcd6p antibodies and anti-HA antibodies. Lanes 1 and 2 contain samples from the WT strain bearing the empty vector. All Western blotting was carried out two or more times, and representative results are presented for the *ccr4*Δ (A) and *not5*Δ (B) mutants. All results are summarized in Table 5. (C) Most mutants with lowered levels of HA$_3$-Gcn4p, such as the *not5*Δ strain, maintain their SM^s phenotypes after transformation with the high-copy-number *GCN4-HA$_3$* plasmid (2μm). The *not5*Δ and *gcn4*Δ mutants and the WT strain containing p2382 (*CEN*), pHQ1239 (2μm), or empty vector were replica plated to SC−ILV medium containing 1 μg of SM per ml and incubated at 25°C for 3 days.

Gcn4p interacts with the RSC and CCR4-NOT complexes dependent on hydrophobic residues in the activation domain. Previously, we showed that SAGA, SWI/SNF, and SRB/MED in WCEs bind specifically to a full-length GST-Gcn4p fusion, dependent on clusters of hydrophobic amino acids in the activation domain required for activation by Gcn4p in vivo (29, 30, 54, 93). Here, we performed similar experiments probing for subunits in the complexes newly determined to be required for Gcn4p activity: RSC, CCR4-NOT, and the Paf1 complex. Sth1p, the catalytic subunit of RSC, coprecipitated with WT GST-Gcn4p but not with the mutant protein containing 10 alanine substitutions in four hydrophobic clusters (10Ala) that destroy activation by Gcn4p in vivo (54) (Fig. 7A, lanes 3 and 4). Similar results were obtained by probing for Swp73p of SWI/SNF and Srb7p of SRB/MED. As described above, Rsc1p and Rsc2p form mutually exclusive RSC complexes. To determine whether Gcn4p interacts with both forms of RSC, we used extracts from the *rsc1*Δ and *rsc2*Δ strains in pull-down assays. The Sth1p subunit in both extracts was precipitated by the WT but not by 10Ala GST-Gcn4p (Fig. 7A, lanes 7 and 8 and lanes 11 and 12, respectively). Moreover, pull-down assays using extracts from strains in which either Rsc1p or Rsc2p is tagged with 13 copies of the Myc epitope show that WT GST-Gcn4p can precipitate both Rsc1p and Rsc2p (Fig. 7B, lane 3). We conclude that both forms of the RSC complex can interact specifically with the Gcn4p activation domain in vitro, consistent with our data showing that both forms of RSC are required for WT activation of various Gcn4p-dependent promoters (Fig. 4).

The results of pull-down assays shown in Fig. 7C and D suggest that CCR4-NOT also interacts specifically with the Gcn4p activation domain but that the Paf1 complex does not. Probing the pull-down assays from a WT extract with antibodies against the Caf16p subunit of CCR4-NOT revealed a higher level of binding to the WT than to 10Ala GST-Gcn4p or GST alone, comparable to the results obtained for the SRB/

TABLE 5. Summary of HA$_3$-Gcn4p levels in Gcn$^-$ mutants

Mutant allele	HA$_3$-Gcn4p level (% of WT)[a]		Complementation[b] of SM^S phenotype by 2μm/*GCN4-HA$_3$*
	With *CEN*/*GCN4-HA$_3$*	With 2μm/*GCN4-HA$_3$*	
*ada1*Δ	40	100	No
*ada2*Δ	250		
*ada3*Δ	200		
*ada5*Δ	40	100	No
*bdf1*Δ	40	200	Partial
*caf1*Δ	100		
*ccr4*Δ	250		
*dhh1*Δ	20	200	No
*gal11*Δ	100		
*gcn5*Δ	300		
*hpr1*Δ	40	100	No
*med2*Δ	100		
*not5*Δ	40	150	No
*paf1*Δ	100		
*pgd1*Δ	100		
*rox3*Δ	100		
*rsc2*Δ	100		
*snf5*Δ	150		
*snf6*Δ	100		
*spt7*Δ	100		
*srb10*Δ	100		
*srb2*Δ	100		
*srb5*Δ	100		
*swi2*Δ	400		
*swi3*Δ	40	100	Partial
*swp73*Δ	250		
*tfg3*Δ	120		

[a] The level of HA$_3$-Gcn4p was measured by Western analysis of two or more independent transformants of the relevant deletion strains containing *GCN4-HA$_3$* on single-copy (*CEN*) or high-copy-number (2μm) plasmids (as shown in Fig. 5A and B) and expressed as a percentage of the level measured in WT cells containing *GCN4-HA$_3$* on the single-copy plasmid.

[b] Summary of the results of all experiments of the kind shown in Fig. 5C.

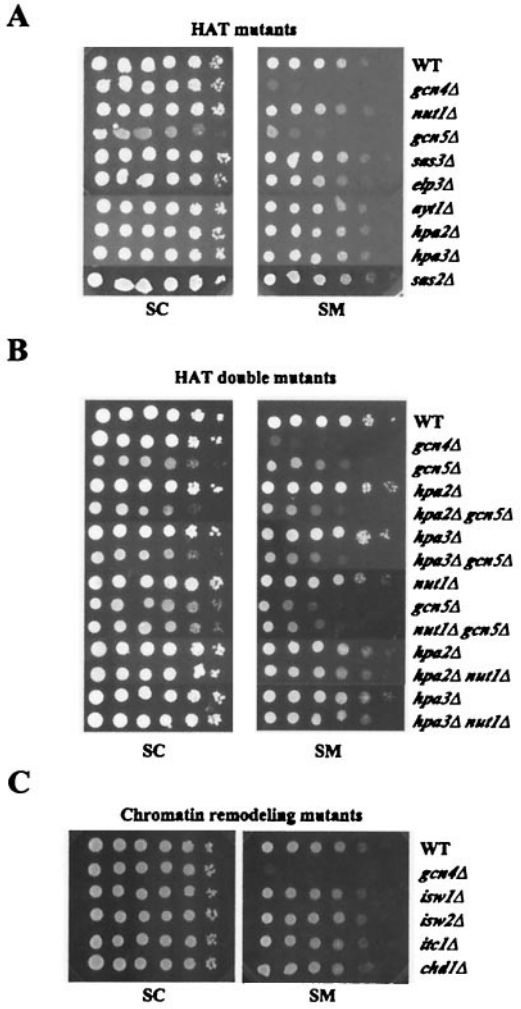


FIG. 6. Deletions of chromatin-modifying enzymes do not generally produce Gcn$^-$ phenotypes (A to C). The indicated mutant and WT strains were tested for sensitivity to SM as shown in Fig. 1A.

MED subunit Srb7p (Fig. 7C, lanes 2 to 4). As expected, the TFIID subunit Taf40p did not precipitate with GST-Gcn4p (30, 93). Similarly, using extracts from strains expressing Myc_{13}-tagged forms of Dhh1p or Ccr4p in pull-down assays, we observed specific binding by both of these CCR4-NOT subunits to WT GST-Gcn4p (Fig. 7D, lanes 1 to 8). By contrast, Myc_{13}-tagged Paf1p did not interact with GST-Gcn4p dependent on the activation domain (Fig. 7D, lanes 11 and 12). As Ccr4p is a component of CCR4-NOT and the Paf1 complex, it most likely precipitates with GST-Gcn4p as a component of the former complex.

Gcn4p recruits the coactivator complexes required for full activation function to a single promoter in vivo. The results above indicate that SAGA, SWI/SNF, RSC, SRB/MED, CCR4-NOT, and Paf1 complexes and Mbf1p all play a role in activation by Gcn4p in vivo. To determine whether each of these coactivators is physically recruited by Gcn4p to one of its target genes, *ARG1*, we performed ChIP experiments. A panel of 15 strains was constructed from the *gcn4*Δ mutant (strain 249), each containing a different coactivator subunit tagged with 13 Myc epitopes and transformed with either the *GCN4* plasmid pHQ1239 or an empty vector. An additional strain was produced by introducing a plasmid expressing Myc_{13}-tagged Gcn4p in the *gcn4*Δ strain. None of the epitope-tagged strains exhibited any growth phenotypes that would signify an effect of the Myc_{13} tag on coactivator function (data not shown). Cells

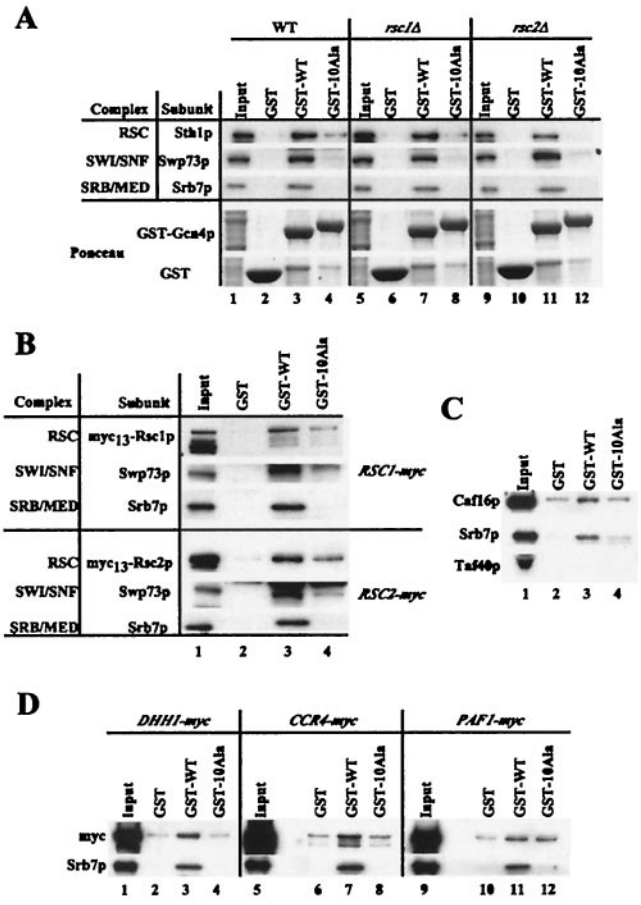


FIG. 7. GST-Gcn4p interacts specifically with the RSC and CCR4-NOT complexes but not with the Paf1 complex in cell extracts. Equal amounts of GST, GST-Gcn4p (GST-WT), and GST-Gcn4p containing 10 alanine substitutions in the activation domain (GST-10Ala) were incubated with WCEs from yeast strains grown in YPD medium. The GST proteins were precipitated with glutathione Sepharose, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and detected by Western analysis. (A) Equal amounts of WCEs from WT (BY4741), *rsc1*Δ (strain 4686), and *rsc2*Δ (strain 5266) strains were incubated with the GST proteins. Blots were probed with antibodies against the proteins listed on the left of the upper three panels. The bottom two panels depict Ponceau S staining of total proteins. Input lanes contained 1% of the WCEs used in pull-down assays. (B) Same as panel A except that WCEs were from derivatives of WT strain BY4741 expressing Myc_{13}-Rsc1p (top three panels) or Myc_{13}-Rsc2p (lower three panels), detected with Myc antibodies. (C) GST proteins were incubated with yeast WCE from WT strain BY4741, and the pull-down assays were probed with antibodies against the proteins listed beside the three panels, subunits of CCR4-NOT (Caf16p), SRB/MED (Srb7), and TFIID (TAF11/TAF40). (D) The GST proteins were incubated with WCEs from strains derived from BY4741 expressing Myc_{13}-tagged forms of Dhh1p (*DHH1-myc*), Ccr4p (*CCR4-myc*), or Paf1p (*PAF1-myc*). The tagged proteins were detected with Myc antibody, and SRB/MED interaction was detected with Srb7p antibody. Input lanes contained 10% of the WCEs used in the pull-down assays.

were induced with SM, and the tagged proteins were immunoprecipitated with Myc antibody. The relative amount of precipitating DNA containing the *ARG1* UAS or the *POL1* open reading frame (ORF) (analyzed as a negative control) was determined by quantitative PCR (Fig. 8A). The amounts of precipitated *ARG1* DNA, after normalization for the amount of precipitating *POL1* DNA, in the WT and *gcn4*Δ strains were compared in three independent experiments. The results are presented in Fig. 8B and C as the average ratios of precipitated *ARG1* DNA in WT versus *gcn4*Δ cells. As expected, Myc_{13}-Gcn4p bound specifically to the *ARG1* promoter in cells starved with SM (Fig. 8A, lanes 1 and 2). Strains with tagged subunits unique to SWI/SNF (Swi2p), SAGA (Spt7p), or SRB/MED (Srb6p) all showed a strong dependence on Gcn4p for immunoprecipitation of the *ARG1* UAS, with *GCN4*/*gcn4*Δ ratios of 7.5 or greater. Similar results were obtained for Gal11p and Sin4p, which reside in both SRB/MED and the Paf1 complex (Fig. 8B). Less pronounced but significant Gcn4p-dependent immunoprecipitation of the *ARG1* UAS was observed for the strains containing Myc_{13}-Mbf1p or Myc_{13}-tagged subunits of the Paf1 complex (Paf1p) and CCR4-NOT (Ccr4p and Not2p) (*P* values of 0.02, 0.04, 0.06, and 0.014, respectively, in an unpaired Student's *t* test) (Fig. 8A and C). The Gcn4p-dependence of *ARG1* UAS immunoprecipitation was not statistically significant for any of the remaining tagged strains in Fig. 8C when considered individually. However, significant Gcn4p dependence was observed when all of the data from strains with tagged Not2p, Dhh1p, Not5p, or Caf1p were analyzed as a group representing the CCR4-NOT complex (*P* value of 0.05; $P = 0.007$ with Myc_{13}-Ccr4p results also included). Similarly, significant Gcn4p-dependence of *ARG1* UAS immunoprecipitation was observed when the data from strains with tagged Rsc8p and Sth1p were analyzed as a group representing RSC (*P* value of 0.03). We observed no *GCN4*-dependent immunoprecipitation of the *ARG1* UAS in a number of other strains containing Myc_{13}-tagged Tho2p, Rpn6p, or Rpn11p (data not shown), supporting the specificity of our findings on Gcn4p-dependent recruitment of coactivator subunits.

The fact that certain tagged subunits of CCR4-NOT and RSC showed weaker Gcn4p-dependent binding to *ARG1* than others may indicate that the former subunits are not efficiently cross-linked to chromatin and dissociate from the rest of the chromatin-bound complexes during immunoprecipitation. Alternatively, the Myc_{13} epitopes on these subunits may be inaccessible to antibodies in the initiation complexes formed at *ARG1* by Gcn4p. The greater Gcn4p-dependent association of SRB/MED, SWI/SNF, and SAGA with *ARG1* (Fig. 8B) compared to that seen for Mbf1p, CCR4-NOT, the Paf1 complex, and RSC (Fig. 8C) could indicate that relatively larger amounts of the first three complexes are recruited by Gcn4p to the *ARG1* promoter. Alternatively, the last three complexes and Mbf1p may undergo relatively higher levels of Gcn4p-independent binding at *ARG1*. The former explanation is favored by the fact that the absolute amounts of precipitated *ARG1* DNA (normalized for *POL1* DNA precipitation) for Mbf1p, CCR4-NOT, the Paf1 complex, and RSC subunits were relatively smaller in the *GCN4* strains than the amounts that precipitated with the SRB/MED, SWI/SNF, and SAGA subunits (Fig. 8A and data not shown). However, it is possible that comparable amounts of all six coactivators are recruited to the promoter

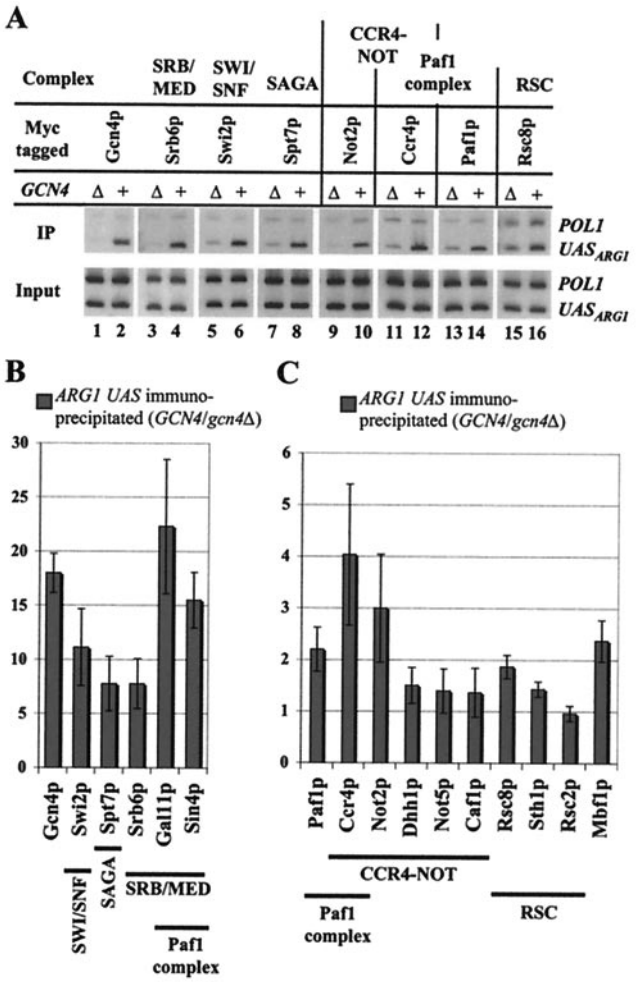


FIG. 8. Evidence from ChIP analysis that Gcn4p recruits seven coactivators to the *ARG1* promoter in vivo. (A) *gcn4*Δ strains expressing different Myc-tagged coactivator subunits, all derived from the *gcn4*Δ strain 249, were transformed with the high-copy-number *GCN4* plasmid pHQ1239 or empty vector. The resulting transformants and *gcn4*Δ strain 249 containing vector or the *GCN4-myc* plasmid pSK-1 were induced with SM and treated with formaldehyde. Chromatin was sheared, heated to reverse the cross-links, and immunoprecipitated with anti-Myc antibodies. The amounts of coprecipitated DNA containing the *ARG1* UAS or *POL1* ORF (upper panels [IP]) and the corresponding amounts in the input chromatin samples (lower panels) were measured by quantitative PCR. Representative results are shown in lanes 3 to 16 for the high-copy-number *GCN4* (even-numbered lanes) and vector (odd-numbered lanes) transformants of strains containing the indicated Myc_{13}-tagged subunits. Lanes 1 and 2 show representative results from the *gcn4*Δ strain transformed with vector or the *GCN4-myc* plasmid. (B and C) For each ChIP experiment, the PCR products were quantified by phosphorimaging analysis, and the ratio of UAS_{ARG1} signals in the immunoprecipitated samples to those in the input samples was calculated and normalized for the corresponding ratio calculated for the *POL1* signals. The resulting normalized ratio $(IP_{UASARG1}/input_{UASARG1})/(IP_{POL1}/input_{POL1})$ obtained for the *GCN4* strain was divided by the corresponding normalized ratio calculated for the *gcn4*Δ strain. The resulting values obtained in three or more independent experiments for each tagged strain were averaged, and the mean values and standard errors were plotted in the histograms as the ratios of the amounts of UAS_{ARG1} specifically associated with each tagged subunit in *GCN4* versus *gcn4*Δ cells.

and that Mbf1p, CCR4-NOT, the Paf1 complex, and RSC dissociate more rapidly, or travel with RNA PolII into the *ARG1* coding region. At odds with the last possibility, we did not see significant Gcn4p-dependent precipitation of *ARG1* coding sequences when we used primers complementary to the 3′ end of the ORF in any of the ChIP assays whose results are shown in Fig. 8 (data not shown). Thus, detectable Gcn4p-dependent binding of all six coactivator complexes was limited to the *ARG1* 5′ noncoding region.

DISCUSSION

Gcn4p requires and recruits a multiplicity of coactivators for transcriptional activation in vivo. Several genetic screenings were conducted previously to identify gene products required for transcriptional activation of amino acid biosynthetic genes by Gcn4p (28, 93, 113, 137), but none had saturated this Gcn$^-$ class of mutants. We used strains from the *Saccharomyces* Genome Deletion Project to conduct a more systematic analysis of nonessential genes implicated previously in transcriptional control for their involvement in activation by Gcn4p. We defined a Gcn$^-$ mutant as one showing sensitivity to Ile/Val starvation imposed by SM, indicating reduced expression of one or more *ILV* genes, and also diminished activation of the UAS_{GCRE}-*CYC1-lacZ* reporter, which is wholly dependent on Gcn4p. We identified 27 such mutants that showed reductions of >25% in resistance to SM and >35% in levels of UAS_{GCRE}-*CYC1-lacZ* expression relative to WT levels (Table 1). We showed that most of these Gcn$^-$ mutants were also defective for induced expression of a *HIS3-GUS* reporter or one of the authentic Gcn4p target genes *SNZ1*, *HIS4*, *ILV2*, and *ARG1* (Tables 3 and 4), consistent with a defect in transcriptional activation by Gcn4p. These additional assays revealed that most coactivator mutations do not impair activation of every Gcn4p-dependent promoter we examined. Indeed, we identified three additional mutants that were defective for transcriptional activation of *HIS3-GUS* and one or more authentic Gcn4p target genes but did not show strong sensitivity to SM or impaired induction of UAS_{GCRE}-*CYC1-lacZ* reporter, namely, *mbf1*Δ, *spt3*Δ, and *rsc1*Δ. It is noteworthy that transcriptional induction of *SNZ1*, *HIS4*, *ILV2*, and *ARG1* was virtually unaffected by deletion of Gcn5p, perhaps the best-characterized coactivator for Gcn4p. Thus, there is a strong precedent for the promoter specificity exhibited by the new coactivators identified here.

In addition to a defect in Gcn4p activation, a Gcn$^-$ phenotype could result from an impairment of core promoter function, reduced activity of a different required activator, or a defect in transcriptional elongation through the coding sequences of the genes under study. By considering the relative effects of the Gcn$^-$ mutations on induced versus uninduced expression of reporter constructs and authentic mRNAs, we identified mutants in which the phenotype could be attributed at least partly to impaired activation by Gcn4p. Figure 9 summarizes these results (data diagnostic of a Gcn4p activation defect are highlighted in dark green). Inspection of these data shows that mutations in the following 29 coactivator proteins impaired Gcn4p-mediated activation of one or more promoters: (i) Mbf1p, (ii) seven of the eight SAGA subunits examined, (iii) five of the six SWI/SNF subunits examined, (iv) Rsc1p and Rsc2p of RSC, (v) Not5p, Caf1p, Dbf2p, Dhh1p, and Ccr4p of CCR4-NOT, (vi) Srb9p, Srb10, Srb5p, Med2p, Pgd1p, Rox3p, and Gal11p of SRB/MED, (vii) Cdc73 of the Paf1 complex, and (viii) Hpr1p of THO/TREX and the Paf1 complex. Less definitive results were obtained for mutations in Tfg3p and five other subunits of CCR4-NOT (Caf4p, Caf16p, Caf17p, Caf40p, and Caf130p) (Fig. 9). Thus, in addition to confirming previous findings that SWI/SNF, SAGA, SRB/MED, and Mbf1p are required for WT activation by Gcn4p, our results implicate CCR4-NOT, RSC, and the Paf1 complex in this process. Moreover, they reveal which nonessential subunits in each of these complexes are crucial for activation by Gcn4p, allowing future genetic studies to focus on these key proteins.

Recombinant Gcn4p can interact with the SAGA, SWI/SNF, SRB/MED, and NuA4 complexes, present in cell extracts or in purified form, dependent on hydrophobic clusters in the activation domain (30, 93, 98, 132). In addition, the DNA binding domain of Gcn4p binds specifically to Mbf1p in vitro (127). Here we showed that Gcn4p can also interact specifically with CCR4-NOT and both forms of RSC (containing Rsc1p or Rsc2p) in cell extracts.

Previous ChIP experiments provided strong evidence that Gcn4p recruits Gcn5p to the *HIS3* promoter in living cells (63, 64). Our ChIP data showing Gcn4p-dependent binding of Spt7p to the *ARG1* promoter are consistent with the idea that Gcn4p recruits Gcn5p as a component of SAGA. It was also shown previously that Gcn4p recruits the Snf5p subunit of SWI/SNF to a modified *PHO5* promoter containing a UAS_{GCRE} (126). Here, we confirmed that Gcn4p recruits Swi2p/Snf2p to the authentic target gene *ARG1*. ChIP results reported by Park et al. indicated that Rgr1p is recruited to the *HIS4* promoter in vivo (98); however, it was unknown whether Gcn4p or another activator functioning at this promoter (Bas1p, Bas2p, or Rap1p) was responsible for recruiting this shared subunit of the SRB/MED and Paf1 complexes. Our ChIP data demonstrate high-level recruitment of the SRB/MED-specific subunit Srb6p by Gcn4p to the *ARG1* promoter (Fig. 8).

We also observed a lower level of recruitment of Mbf1p, several subunits of CCR4-NOT, Rsc8p and Sth1p of RSC, and Paf1p by Gcn4p to the *ARG1* promoter (Fig. 8). Thus, it appears that Gcn4p recruits both SRB and Paf1 mediators to the same promoter, as reported for other activators (102). It also recruits two different ATP-dependent chromatin-remodeling complexes, RSC and SWI/SNF, and two different coactivators that function at least partly as adaptors for TBP (SAGA and Mbf1p). Except for the Paf1 complex, all of these coactivators showed specific binding to Gcn4p in WCEs and hence may be recruited to *ARG1* through direct contact with the Gcn4p activation domain. Perhaps the Paf1 complex is recruited indirectly by Gcn4p through promoter-bound RNA PolII. Considering that the Paf1 complex was found to be associated with promoters and coding regions of various yeast genes while SRB/MED was restricted to the promoters, it is possible that the Paf1 complex is exchanged for SRB/MED during the transition from initiation to elongation (102). However, we could not detect binding of Paf1p to *ARG1* coding sequences and found it only in the 5′ noncoding region of *ARG1*.

Although Gcn4p recruits seven different coactivators to the

Relevant Genotype	Cofactor Complex	SM^R	Induced *UAS GCRE - CYC1-lacZ*	Induced *HIS3-GUS*	Induced *SNZ1*	Induced *HIS4*	Induced *ARG1*	Induced *ILV2*
WT	NA	100	100	100	100	100	100	100
gcn4Δ	activator	5	1	11	12	31	14	32
gcn4Δ/gcn4Δ	activator	5	0.2		16	23	7	26
mbf1Δ	NA	100	180	27	63	73	77	68
ada2Δ	ADA, SAGA	51	22	16	70	43	96	
ada3Δ	ADA, SAGA	46	30	20				
gcn5Δ	ADA, SAGA	41	29	6	124	98	143	71
ada1Δ	SAGA	26	5.1	20	47	29	101	59
ada5Δ	SAGA	26	3.1	32	45	27	106	45
spt3Δ	SAGA	89	100	29	68	52	86	
spt7Δ	SAGA	24	25	9.1	41		121	
spt8Δ	SAGA	95	110	84				
tfg3Δ	Multiple	28	22	58				
swi2Δ	SWI/SNF	35	40	169	44	206	118	109
swi3Δ	SWI/SNF	62	33	25				
snf5Δ	SWI/SNF	55	28	117				
snf6Δ	SWI/SNF	55	27	82				
snf11Δ	SWI/SNF	103	61	83				
swp73Δ	SWI/SNF	41	39	129	30	32		
rsc1Δ	RSC	92	100	4.2	67	88	67	70
rsc2Δ	RSC	70	42	49	144	63	111	124
not3Δ	CCR4-NOT	100	140	52				
not4Δ	CCR4-NOT	100	100	51				
not5Δ	CCR4-NOT	42	17	39	155	74	72	79
caf1Δ	CCR4-NOT	27	8.2	10	76	77	48	62
caf4Δ	CCR4-NOT	97	110	54				
caf16Δ	CCR4-NOT	100	110	40				
caf17Δ	CCR4-NOT	91	80	64				
caf40Δ	CCR4-NOT	100	120	33				
caf130Δ	CCR4-NOT	100	110	46				
dbf2Δ	CCR4-NOT	70	93	20				
dhh1Δ	CCR4-NOT	64	6.3	12	65	102	41	56
srb9Δ	SRB/MED, CCR4-NOT	84	69	12				
srb10Δ	SRB/MED, CCR4-NOT	58	35	73				
srb11Δ	SRB/MED, CCR4-NOT	92	91	77				
srb5Δ	SRB/MED	73	47		126	118	148	92
med2Δ	SRB/MED	36	32	43	65	84	83	88
pgd1Δ	SRB/MED	58	26	259	62	67	57	72
rox3Δ	SRB/MED	12	35	21	38	67	52	62
sin4Δ	SRB/MED, Paf1 complex	85	100	119	68	94	85	86
gal11Δ	SRB/MED, Paf1 complex	24	27	55	55	102	97	78
cdc73Δ	Paf1 complex	94	92	42	98	84	100	77
paf1Δ/paf1Δ	Paf1 complex	50	26		97	126	149	136
ccr4Δ	CCR4-NOT, Paf1 complex	36	4.3	10	57	49	44	51
hpr1Δ	Paf1 complex, THO/TREX	27	1.3	3.3	49	50	66	125

FIG. 9. Summary of effects of coactivator deletions on induction by Gcn4p of multiple target genes. The table includes only those mutants for which we assayed expression of *HIS3-GUS* or Gcn4p target gene transcripts by Northern analysis. The data shown here are from Table 1 (SM^r and induced UAS_{GCRE}-*CYC1-lacZ* expression), Table 3 (induced *HIS3-GUS* expression), and Table 4 (induced *SNZ1*, *HIS4*, *ARG1*, and *ILV2* mRNAs). Data that are in italics in the tables are highlighted in red here; those underlined and in boldface with an asterisk, underlined and in boldface, or underlined only in the tables are shown in dark green, light green, or yellow, respectively. Briefly, data highlighted in dark and light green provide strong and suggestive evidence, respectively, that Gcn4p activation of the promoter is impaired by the mutation. Data highlighted in yellow indicate reduced promoter function in the mutant that cannot be attributed specifically to a defect in activation by Gcn4p. Data highlighted in red indicate greater-than-WT expression of the promoter in the mutant.

ARG1 promoter, it does not seem to require SAGA or SWI/SNF for WT activation of this promoter (Fig. 9). As noted above, SAGA may have offsetting positive and negative functions at *ARG1*, being required for activation by Gcn4p and for arginine-mediated repression by the ArgR/Mcm1p repressor. Nevertheless, given the idiosyncratic coactivator requirements at different Gcn4p target genes (Fig. 9), it is likely that Gcn4p frequently recruits more coactivators to a given target gene than are needed for high-level induction of that promoter. Because it activates hundred of genes (68, 94), Gcn4p may have evolved to interact effectively with many coactivators to counteract a wide range of repressive chromatin structures and sequence-specific repressors throughout the genome, and also to provide redundant pathways for recruitment of TBP, GTFs, and RNA PolII.

Gcn4p requires subunits of the HAT complex SAGA for transcriptional activation in vivo. Our finding that WT activation of the UAS_{GCRE}-*CYC1-lacZ* and *HIS3-GUS* reporters by Gcn4p requires the HAT Gcn5p agrees with previous results showing impaired induction of certain Gcn4p target genes (*HIS3*, *ILV1*, and *TRP3*) in a *gcn5* mutant (40). By contrast, we and others found that *HIS4*, *SNZ1*, *ARG1*, and *ILV2* transcription is Gcn5p independent (Fig. 9), and there is evidence that the Gcn5p requirement at *HIS3* can be diminished by changes in the Gcn4p binding site (40). Since UAS_{GCRE}-*CYC1-lacZ* contains tandem copies of a Gcn4p binding site from *HIS4* and

is clearly Gcn5p dependent (Fig. 2), other elements in the *HIS4* promoter may reduce its Gcn5p dependence compared to other Gcn4p target genes like *HIS3*.

We found that the nonessential subunits of SAGA, Ada2p, Ada3p, Ada1p, Ada5p/Spt20p, and Spt7p are required for WT activation of multiple promoters by Gcn4p in vivo (Fig. 9), consistent with previous reports on *ada2* (10), *ada3* (100), *ada5*/*spt20* (80), and *ada1* (52) mutants. Overall, the deletions of *SPT7*, *ADA5*/*SPT20*, and *ADA1* had greater effects on Gcn4p activation of multiple promoters than did deletion of *ADA2*, *ADA3*, *GCN5*, or *SPT3*, whereas deleting *SPT8* had little or no effect (Fig. 9). Similar results were reported previously concerning the relative effects of *gcn5*Δ, *spt7*Δ, *ada5*Δ/*spt20*Δ, *spt3*Δ, and *spt8*Δ mutations on *HIS3* activation by Gcn4p (8). Moreover, others have shown that *spt7*Δ, *ada5*Δ/*spt20*Δ, and *ada1*Δ mutants display a broader range and severity of growth phenotypes compared to *gcn5*Δ, *ada2*Δ, *ada3*Δ, *spt3*Δ, and *spt8*Δ mutants (52, 80, 119), which may be attributable to a requirement for Spt7p, Ada5p/Spt20p (42), and Ada1p (119) for SAGA integrity. These and other genetic findings indicate that SAGA performs an important function beyond the HAT activity of Gcn5p, such as TBP recruitment (34, 67, 78, 108, 119).

We observed higher-than-WT expression of the UAS_{GCRE}-*CYC1-lacZ* and *HIS3-GUS* reporters in *spt3*Δ and *spt8*Δ mutants under noninducing conditions (Tables 1 and 3), in accordance with previous observations that *HIS3* and *TRP1* mRNA levels are elevated in such mutants (8). The negative function of SAGA at the latter genes has been attributed to inhibition of TBP binding. At *ARG1*, Gcn5p-dependent histone H3 acetylation, most likely in the context of SAGA, is required for transcriptional repression by ArgR/Mcm1p and is correlated with reduced TBP binding to the promoter (107).

The smaller HAT complex known as ADA shares Ada2p, Ada3p, and Gcn5p with SAGA, but ADA uniquely contains Ahc1p. The deletion of *AHC1* had no effect on activation of the *ILV* genes (judging from the WT SM resistance of the mutant or UAS_{GCRE}-*CYC1-lacZ* (Table 1), consistent with the finding that purified ADA does not interact with Gcn4p in vitro (132). Because the ADA complex is unstable in the absence of Ahc1p (32), it seems likely that Ada2p, Ada3p, and Gcn5p promote activation by Gcn4p in the context of SAGA and not ADA.

Mutants lacking any one of seven nonessential HATs besides Gcn5p had little or no defect in activation of *ILV* genes by Gcn4p, as judged by their WT resistance to SM, including the HATs found in the SRB/MED and NuA3 coactivators, Nut1p and Sas3p, respectively (Fig. 6A). Even in strains lacking Gcn5p, we observed no increase in SM sensitivity upon deleting Nut1p (Fig. 6B). As the *gcn5*Δ *sas3*Δ strain is inviable, these two HATs could make overlapping contributions to activation by Gcn4p. Indeed, the modest effects of the *sas3*Δ and *yer049*Δ mutations on UAS_{GCRE}-*CYC1-lacZ* induction (Table 1) are consistent with a minor role for NuA3 in activation of this reporter. However, neither mutant has an SM^s phenotype, and Gcn4p could not recruit NuA3 to chromatinized templates in vitro (132).

In vitro, Gcn4p can interact with NuA4, containing the essential HAT Esa1p (132). In addition, depletion of Esa1p in vivo reduced histone H4 acetylation at *HIS3* and *HIS4*, along with other promoters analyzed in parallel. Except for ribosomal protein genes, however, Esa1p depletion was not associated with reduced transcription. Consistently, we observed no SM^s phenotype in temperature-sensitive *esa1* mutants at semipermissive growth temperatures (data not shown). Therefore, Gcn5p is the only HAT with an established in vivo function in transcriptional activation by Gcn4p.

Gcn4p requires SWI/SNF and RSC but not the ISWI chromatin-remodeling factors for transcriptional activation at certain promoters in vivo. We confirmed our previous finding that Gcn4p requires the ATP-dependent chromatin-remodeling complex SWI/SNF for transcriptional activation in vivo. Except for *SNF11*, deletions of all SWI/SNF subunits produced SM^s phenotypes and defects in UAS_{GCRE}-*CYC1-lacZ* induction comparable to those observed in the *swi2*Δ/*snf2*Δ mutant, which lacks the ATPase subunit. It was shown that deletions of *SWI2*/*SNF2*, *SWI3*, *SNF5*, or *SNF6* affect the integrity of the SWI/SNF complex; however, Swi2p/Snf2p was still found in a high-molecular-weight complex of ca. 1 MDa that lacked Swi3p, Snf5p, and Snf6p in these mutants (99). Interestingly, the Swi2p/Snf2p-containing subcomplex present in a *snf5*Δ mutant was defective for binding to the *SUC2* promoter in vivo, implicating Snf5p (or one of the other subunits lacking in this subcomplex) in recruitment of SWI/SNF by activators. Consistently, Gcn4p was cross-linked to Snf5p, Swi1p, and Swi2p/Snf2p in vitro (95), suggesting that interactions with multiple noncatalytic subunits may contribute to SWI/SNF recruitment by Gcn4p.

It was surprising that only Swi3p was required for activation of *HIS3-GUS* (Table 3), particularly since it was shown previously that transposon insertions in *SWI2*/*SNF2*, *SWI1*, and *SWP73* all impaired induction of this reporter by Gcn4p in another strain background (93). Perhaps the deletion library background contains a genetic modifier of the activation defects conferred by certain *swi*/*snf* mutations. Mutations in Swi2p, Snf5p, and Swp73 did result in derepressed *HIS3-GUS* expression (Table 3), and the *swi2*Δ/*snf2*Δ strain had derepressed *HIS4* mRNA levels (Table 4) under noninducing conditions. Thus, activation defects in these mutants may be obscured by offsetting defects in a repression mechanism.

Our results showed that the Rsc2p subunit of RSC is required for full activation by Gcn4p of UAS_{GCRE}-*CYC1-lacZ*, *HIS3-GUS*, and *HIS4*, whereas activation of *HIS3-GUS*, *SNZ1*, and *ARG1* was reduced by deletion of *RSC1* (Fig. 9). Rsc2p is more abundant than Rsc1p (16), and we found that a 2μm/*RSC1* plasmid can suppress the SM^s phenotype of the *rsc2*Δ strain (data not shown). Thus, the SM^s phenotype of *rsc2*Δ cells may reflect the fact that the Rsc1p complex is not abundant enough to support full activation by Gcn4p at one of the *ILV* promoters. However, the strong defect in *HIS3-GUS*, *SNZ1*, and *ARG1* activation in *rsc1*Δ cells suggests that the Rsc1p complex is required to alter the chromatin structure at these genes in a way that cannot be performed by the Rsc2p complex.

It may seem surprising that Gcn4p requires both SWI/SNF and RSC for full activation of certain promoters, including UAS_{GCRE}-*CYC1-lacZ*, *SNZ1*, and *HIS4*. Whereas both complexes are recruited by Hir1p and Hir2p to the *HTA1*/*HTB1* histone genes, SWI/SNF functions as a coactivator while RSC recruitment was correlated with repression (26, 97).

Not all ATP-dependent chromatin-remodeling complexes are required for activation by Gcn4p, however, as deletion of the ATPase subunits of the ISWI complexes and of Chd1p had no effect on activation of *ILV* genes by Gcn4p (Fig. 6C).

Gcn4p shows differential requirements for Gal11 module subunits in SRB/MED. The Gal11 module of SRB/MED, containing Gal11p, Med2p, Pgd1p, and Sin4p, has been implicated as a target of activators through biochemical analysis of mutant mediator complexes. SRB/MED complexes purified from *pgd1*Δ, *med2*Δ, or *gal11*Δ strains are devoid of two or all three of these subunits, and they exhibit quantitative reductions in transcriptional activation by Gcn4p in vitro (71, 88, 98). Consistently, the purified *pgd1*Δ mediator complex, lacking Gal11p and Med2p, failed to bind recombinant Gcn4p, and both recombinant Gal11p and Pgd1p can interact with Gcn4p in vitro. However, apart from the *med2* insertion described previously (93), deletions of *GAL11*, *PGD1*, and *MED2* were reported to have little or no effect on activation by Gcn4p in vivo (88, 98). By contrast, in the strain background employed here, deletions of all three genes led to significant defects in activation by Gcn4p. Thus, our genetic data support the idea that the Gal11 module provides an important binding site for Gcn4p in SRB/MED.

The purified *sin4*Δ mediator is devoid of all four Gal11 module subunits (27) and was shown to be more defective than the SRB/MED complexes purified from *pgd1*Δ, *med2*Δ, or *gal11*Δ strains in promoting transcriptional stimulation by Gcn4p in vitro (88). These results suggested that Sin4p is critically required for activation by Gcn4p. Surprisingly, transcriptional activation of *HIS3* (56), *HIS3-GUS*, UAS_{GCRE}-*CYC1-lacZ*, *HIS4*, *ARG1*, and *ILV2* (Fig. 9) was not diminished in the *sin4*Δ mutant. To account for this discrepancy between in vitro and in vivo findings, it could be proposed that the absence of Gal11p, Pgd1p, and Med2p from the *sin4*Δ mediator is an artifact of purification that does not prevail in vivo and that the latter proteins can support strong activation by Gcn4p in the absence of Sin4p in SRB/MED (89). Another possibility is that Sin4p harbors both positive and negative regulatory functions, and elimination of its negative function can overcome the absence of the entire Gal11 module at certain promoters in the context of in vivo chromatin structures. Indeed, the *sin4*Δ mutation led to derepression of UAS_{GCRE}-*CYC1-lacZ*, *HIS3-GUS*, and *ARG1* expression under noninducing conditions (Tables 1, 3, and 4) and was shown to restore partial function to the *CYC1* promoter lacking the native UAS (56).

The activation defects conferred by the *rox3*Δ mutation are comparable to those produced by deletion of *GAL11*, *PGD1*, or *MED2* (Fig. 9); however, Rox3p does not appear to be a component of the Gal11 module (71, 76, 88). Moreover, *ROX3* is an essential gene in other strain backgrounds (89). Perhaps Rox3p provides a second binding site for Gcn4p in SRB/MED. Srb5p and Srb2p, and presumably the subcomplex comprised of these proteins and containing Srb4p and Srb6p (60), also contribute to activation by Gcn4p in vivo (Table 1). There is evidence that Srb5p facilitates a step in gene activation, possibly CTD phosphorylation, subsequent to activator recruitment (71).

Srb10p and Srb11p constitute a kinase-cyclin pair that is thought to impede assembly of the RNA PolII holoenzyme prior to promoter binding through phosphorylation of the CTD (89). Recently, Srb10p was also implicated in rapid degradation of Gcn4p (22). However, Gcn4p target genes were not constitutively induced in an *srb10*Δ mutant (51). Moreover, we observed a Gcn^- phenotype for the *srb10*Δ mutant in our genetic background (Fig. 9). Presumably, the positive effects of Srb10p on Gcn4p function outweigh its role in Gcn4p degradation.

Evidence that the Paf1 complex functions in Gcn4p-mediated transcriptional activation. We found that Paf1p is required for WT SM resistance and induction of the UAS_{GCRE}-*CYC1-lacZ* reporter, whereas the Cdc73p subunit of the Paf1 complex was required for normal induction of the *HIS3-GUS* reporter. However, we observed no defects in activation of authentic Gcn4p target genes in *paf1*Δ or *cdc73*Δ cells (Fig. 9). As the Paf1 complex has been implicated in transcriptional elongation (117), it might be required by Gcn4p only for transcriptional elongation through reporter constructs containing bacterial coding sequences. The same argument could apply to the *hpr1*Δ mutant, shown previously to be defective for transcriptional elongation through *lacZ* (18), as this strain shows very low reporter expression but only a modest defect in activation of authentic Gcn4p target genes (Fig. 9). However, both the *cdc73*Δ and *hpr1*Δ mutants impaired reporter gene expression to a greater extent under inducing than noninducing conditions, suggesting that they contribute to the mechanism of activation by Gcn4p. This observation, plus our finding that Paf1p was recruited by Gcn4p to *ARG1* (Fig. 8), leads us to suggest that the Paf1 complex is a physiological coactivator required by Gcn4p for WT induction of a subset of target genes that harbor transcribed sequences that impede elongation.

Hpr1p belongs to the THO/TREX complex, also involved in elongation, mitotic recombination (18, 19), and nuclear export of mRNA (112, 121). We observed no SM sensitivity in mutants lacking other THO/TREX complex subunits, including Tex1p, Mft1p, and Thp2p (Table 1). However, it is possible that *hpr1*Δ impairs an overlapping function of the Paf1 and THO/TREX complexes in elongation that is critical for activation by Gcn4p. We did not detect any requirement for the Elongator complex or for elongation factors encoded by *SPT4* (Spt complex) or *DST1* (TFIIS) for activation of *ILV* genes by Gcn4p, judging from the WT SM resistance of the corresponding mutants (Table 1). Thus, Gcn4p may depend primarily on the functions of Hpr1p in the Paf1 or THO/TREX complexes for stimulating transcriptional elongation at its target genes.

Gcn4p requires the CCR4-NOT coactivator, which possesses cytoplasmic deadenylase activity. There is a large body of evidence implicating the CCR4-NOT complex in transcriptional activation and repression, including the requirement for Ccr4p in transcriptional activation by Adr1p, its physical association with Srb9p, -10p, and -11p, and its genetic and physical interactions with TBP and associated factors (see the introduction). Our ChIP results provide the first evidence for activator-dependent recruitment of CCR4-NOT to an induced promoter. These results are significant because CCR4-NOT also functions in the cytoplasm as an mRNA deadenylase (130, 131), with Ccr4p itself serving as the catalytic subunit (20, 130). Deadenylation should diminish translation of an mRNA and also render it susceptible to decapping and 5′-3′ exonucleolytic degradation (7, 110). Thus, mutations that merely destroy the

deadenylase activity should not lower transcript levels in the manner we observed for several Gcn4p target mRNAs in *ccr4*Δ cells (Fig. 9). Previously, others provided strong evidence that factors involved in mRNA processing (79) or export from the nucleus (72, 121) become associated with the nascent transcript. Perhaps recruitment of CCR4-NOT by Gcn4p allows subunits of this complex to associate with the nascent transcript and accompany it into the cytoplasm to control deadenylation, translation, and degradation of the mRNA.

MBF1 is required for activation of a subset of Gcn4p-dependent genes. Previous results indicated that Mbf1p serves as an adaptor between the DNA binding domain of Gcn4p and TBP. Deletion of *MBF1* eliminated *HIS3* and *HIS5* mRNAs in vivo, and point mutations in Gcn4p that reduced binding to Mbf1p and a point mutation in Mbf1p that reduced interaction with TBP both lowered *HIS3* mRNA levels (127). The *mbf1*Δ mutation in our strain background did not produce SM sensitivity or impair UAS_{GCRE}-*CYC1-lacZ* expression. However, expression of the *HIS3-GUS* reporter was lower in the *mbf1*Δ strain, in agreement with previous findings, and induction of *SNZ1* and *ILV2* was also impaired. Together, the results indicate a promoter-specific role for Mbf1p in Gcn4p-activated transcription.

Concluding remarks. The seven different coactivators recruited by Gcn4p to *ARG1* could potentially provide a multiplicity of functions important for stimulating transcription initiation. SWI/SNF, RSC, or SAGA could displace or modify nucleosomes to expose binding sites for TBP and RNA PolII in the core promoter. Mbf1p, SAGA, CCR4/NOT, SRB/MED, or the Paf1 complex could help recruit TBP, other GTFs, or RNA PolII to stimulate preinitiation complex assembly. The Paf1 complex or THO/TREX may stimulate the elongation phase of transcription and even promote the export of transcripts from the nucleus (THO/TREX). Given the large size of these coactivator complexes, it seems unlikely that they could all reside at the promoter simultaneously; rather, they may bind transiently and dissociate after carrying out their specific functions at the promoter. Binding of one coactivator may facilitate association of another, e.g., by covalent modification of nucleosomes, augmenting or even replacing direct recruitment by the activator. For example, Swi5p must recruit SWI/SNF to the *HO* gene to permit subsequent binding of SAGA in the absence of activator (24, 61). On the other hand, HAT activity was shown to retain SWI/SNF at promoters in vitro (45) and to target particular nucleosomes for remodeling in vivo (106). In the case of Gcn4p, there is evidence that the bromodomain in Gcn5p is required for transferring SAGA from the activation domain to acetylated nucleosomes, freeing Gcn4p to recruit SWI/SNF (126). It will be interesting to see what other functional interactions exist between the numerous coactivators recruited to promoters by Gcn4p.

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