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A Triad of Subunits from the Gal11/Tail Domain of Srb Mediator Is an In Vivo Target of Transcriptional Activator Gcn4p

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The Srb mediator is an important transcriptional coactivator for Gcn4p in the yeast *Saccharomyces cerevisiae*. We show that three subunits of the Gal11/tail domain of mediator, Gal11p, Pgd1p, and Med2p, and the head domain subunit Srb2p make overlapping contributions to the interaction of mediator with recombinant Gcn4p in vitro. Each of these proteins, along with the tail subunit Sin4p, also contributes to the recruitment of mediator by Gcn4p to target promoters in vivo. We found that Gal11p, Med2p, and Pgd1p reside in a stable subcomplex in *sin4*Δ cells that interacts with Gcn4p in vitro and that is recruited independently of the rest of mediator by Gcn4p in vivo. Thus, the Gal11p/Med2p/Pgd1p triad is both necessary for recruitment of intact mediator and appears to be sufficient for recruitment by Gcn4p as a free subcomplex. The *med2*Δ mutation impairs the recruitment of TATA binding protein (TBP) and RNA polymerase II to the promoter and the induction of transcription at *ARG1*, demonstrating the importance of the tail domain for activation by Gcn4p in vivo. Even though the Gal11p/Med2p/Pgd1p triad is the only portion of Srb mediator recruited efficiently to the promoter in the *sin4*Δ strain, this mutant shows high-level TBP recruitment and wild-type transcriptional induction at *ARG1*. Hence, the Gal11p/Med2p/Pgd1p triad may contribute to TBP recruitment independently of the rest of mediator.

Transcription initiation by RNA polymerase II (Pol II) is dependent on a set of general transcription factors (GTFs), including TATA binding protein (TBP), which recognize the core promoter and facilitate initiation from the correct start site. The stimulation of transcription by activator proteins is dependent on coactivators that serve as adaptors to facilitate recruitment of Pol II and GTFs and also to activate Pol II function. Other coactivators stimulate preinitiation complex (PIC) assembly by remodeling the nucleosome structure of the promoter (21). The Srb mediator is an important coactivator in *Saccharomyces cerevisiae*, consisting of 20 or more distinct polypeptide subunits, which can be found associated with Pol II in a holoenzyme complex (11, 16, 23, 26). Purified mediator can support stimulation of Pol II function by activators in vitro, and it enhances basal transcription and phosphorylation of the C-terminal domain (CTD) of the largest subunit of Pol II (Rpb1p) by the general factor TFIIH (reviewed in reference 30).

The Srb2p and Srb4p-Srb11p subunits of Srb mediator were identified genetically as suppressors of truncations of the Rpb1p CTD (18). Transcription in cell extracts is highly dependent on Srb4p, Srb2p, and Srb6p (35, 41), and a temperature-sensitive *srb4* mutation impairs transcription from most promoters in vivo (42). Thus, these Srb proteins carry out important general functions in transcription initiation (35). A number of mediator subunits were identified genetically by mutations that impair activation or repression of specific genes, implicating Srb mediator in transcriptional regulation in vivo. The remaining mediator subunits were identified biochemically from large-scale purification of a native mediator complex (30).

The mediator can be divided roughly into three modules. The Srb4 module consists of eight subunits (Srb2p, Srb4p to Srb6p, mediator protein 6 [Med6], Med8, Med11, and Rox3p) (15, 17, 23), of which four are essential (30). This module can interact physically with the Rpb1p CTD, TBP, and TFIIB, and it supports basal, but not activated, transcription in vitro (15), although Srb4p itself was identified as a recruitment target for the activator Gal4p (17). It was proposed that the Srb4 module corresponds to the head domain of mediator, as visualized in three-dimensional reconstructions of electron micrographs, which makes several contacts with Pol II in the holoenzyme complex (8). The Med9/Med10 module contains several essential subunits and, together with Rgr1 and Nut1p, has been equated with the middle domain of mediator (8). The Gal11 module, or tail domain, contains Gal11p, Sin4p, Med2p, and Pgd1p/Hrs1p, none of which is essential (30). The tail domain is tethered to the rest of mediator through the Rgr1p CTD (8, 25), and deletion of *SIN4* leads to loss of Gal11p, Med2p, and Pgd1p from the mediator during purification. The last three proteins also were missing from mediator complexes purified from strains lacking only Gal11p, Med2p, or Pgd1p (23, 29, 33); however, dissociation of all three subunits was not observed at early steps in the purification (29). Considering that distinct in vivo phenotypes are associated with single deletions of *GAL11*, *MED2*, or *PGD1* (29, 40), elimination of single subunits of the Gal11p/Med2p/Pgd1p triad probably does not disrupt the entire tail domain in vivo.

There is accumulating evidence that the mediator tail domain is an important target of transcriptional activators. Puri-

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fied mutant holoenzymes lacking Gal11p, Pgd1p, and Med2p are impaired for transcriptional activation by Gal4p, VP16, and Gcn4p, and they fail to bind these activators in vitro. Moreover, all three activators bind to recombinant Gal11p, and Gcn4p also interacts with recombinant Pgd1p in vitro (23, 33). Deletions of internal Gal11p segments that impair binding to Gal4p in vitro produce commensurate activation defects in vivo, consistent with the idea that Gal11p is a direct target for Gal4p. It is unclear whether this is the case for Gcn4p, however, as deletions of Gal11p, Pgd1p (33), or Med2p (29) had little effect on transcriptional activation by Gcn4p in vivo. More recently, however, we observed reductions in the activation of Gcn4p-dependent reporters or target genes in mutants lacking each of these tail subunits (32, 40). Interestingly, a *med10* Ts^- mutation had strong effects on activation by Gcn4p but almost no effect on Gal4p function (12). Thus, Gal4p and Gcn4p may require distinct subsets of mediator subunits for optimal activation in vivo (29).

In this study, we investigated the requirements for tail domain subunits in the interaction of Srb mediator with recombinant Gcn4p in vitro and in the recruitment of mediator subunits by Gcn4p to target promoters in vivo. The in vitro experiments indicate that the four tail domain subunits, Gal11p, Pgd1p, Med2p, and Sin4p, along with the head domain subunit Srb2p, all contribute to a stable interaction of mediator with Gcn4p. By chromatin immunoprecipitation (ChIP) experiments, we found that each of these proteins is also required for high-level recruitment of a mediator head domain subunit by Gcn4p in vivo. Interestingly, Gal11p, Med2p, and Pgd1p reside in a stable subcomplex in *sin4*Δ cells that can bind to Gcn4p in vitro and is recruited by Gcn4p to target genes in vivo independently of the rest of mediator. Finally, we present evidence suggesting that the Gal11p/Med2p/Pgd1p triad can promote TBP recruitment independently of the rest of mediator in *sin4*Δ cells.

MATERIALS AND METHODS

Yeast strains, genetic methods, and plasmids. High-copy-number and single-copy plasmids harboring *GCN4-HA*, pHQ1239, and p2382, respectively, were described previously (40). Yeast strains used in this study are listed in Table 1. Wild-type (WT) and single-deletion mutants from the *Saccharomyces* Genome Deletion Project (10) were purchased from Research Genetics. The mutant alleles were verified previously by PCR amplification of genomic DNA and by complementation of mutant phenotypes with plasmid-borne WT alleles (40). All double mutants were generated by genetic crosses between the corresponding single mutants. The coding sequences for myc_{13} were inserted at the 3′ ends of the coding sequences of various chromosomal genes as a *myc*$_{13}$*-HIS3* cassette, as described previously (40). The presence of the *myc*-tagged alleles was verified by colony PCR and by Western analysis using antimyc antibodies. All of the tagged strains were tested for resistance to sulfometuron-methyl (SM), a sensitive indicator of the extent of transcriptional activation by Gcn4p (40). We observed no decrease in growth on nutrient complete medium and no increase in sensitivity to SM associated with *myc* tagging of *GAL11*, *SRB6*, or *MED2* in strains containing WT alleles of all other mediator subunits and also in the strains harboring deletions of single mediator subunits (data not shown). Thus, it appears that the myc tags on Gal11p, Srb6p, and Med2p have little or no effect on the integrity and function of Srb mediator in vivo. The same was true for the *SIN4-myc* strains except that the *srb2*Δ *SIN4-myc* and *srb5*Δ *SIN4-myc* mutants are more sensitive to SM than are the corresponding *srb2*Δ *SIN4* and *srb5*Δ *SIN4* strains (data not shown). Thus, the myc tag on Sin4p may produce a small reduction in coactivator function when combined with elimination of Srb2p or Srb5p from Srb mediator. Deletion of *GCN4* in the *myc*-tagged strains was carried out with plasmid pHQ1240 and verified as described previously (43).

GST pull-downs, coimmunoprecipitations, Northern blot analysis, and ChIP assays. Bacterial extracts containing glutathione *S*-transferase (GST) proteins and whole-cell extracts (WCEs) from yeast strains grown in yeast extract-peptone-dextrose medium (38) were used for GST pull-down assays as described previously (9) except that yeast cells were broken in a buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM HEPES (pH 7.9), 10 mM $MgSO_4$, 100 mM NH_4SO_4, 12.5 mM potassium acetate, 5 mM EGTA, 2.5 mM dithiothreitol, 0.01% NP-40, 20% glycerol, 1 μg of pepstatin A/ml, 1 mM AEBSF [4-(2-aminoethyl) benzenesulfonyl fluoride], and 2× Complete protease inhibitor without EDTA (Roche), and the lysates were cleared by centrifuging at 16,000 × *g* for 15 min and then for 30 min at 4°C in a microcentrifuge. Coimmunoprecipitation assays were conducted using the same yeast WCEs and antimyc antibodies conjugated to agarose (Santa Cruz Biotechnology). The immune complexes were washed and collected as in GST pull-down assays (9). Rabbit polyclonal antisera used in Western analysis were previously described for Srb7p (13), Med1p (1), Srb2p and Srb5p (41), Tra1p (2), Taf9p (9), Snf5p (4), and Med4p and Med8p (28), as was rat polyclonal antiserum for Rgr1p (22). Taf12p antibodies were kindly provided by J. Reese. Mouse monoclonal antibodies against the myc epitope (9E10) were obtained from Roche. Goat polyclonal antiserum for Ada2p was from Santa Cruz Biotechnology. Hemagglutinin (HA)-tagged proteins were detected with anti-HA monoclonal antibodies from Santa Cruz Biotechnology. Total RNA was extracted and subjected to Northern analysis as described previously (31).

ChIP assays were conducted as previously described (40) with the following modifications. Cultures were grown to optical density at 600 nm of 1.0 to 1.5 in synthetic complete medium (SC) lacking uracil (38). The cells were collected by centrifugation, washed with sterile H_2O, and resuspended in SC lacking uracil, arginine, isoleucine, and valine and containing 0.5 μg of SM/ml. The SM-treated cells were grown for 3 h before adding formaldehyde. Cross-linked chromatin was sonicated for 12 cycles of 30 s at 4°C with at least 30 s cooling on ice per cycle and immunoprecipitated with antibodies against the myc epitope (Roche), TBP (provided by J. Reese), or Rpb1p (18WG16; Abcam, Inc.) as appropriate. The primers used to amplify the *ARG1* upstream activation sequence (UAS), *POL1* open reading frame (ORF) (40), *SNZ1* UAS (43), and *ARG1* TATA element (34) were described previously. The PCR amplification involved denaturation at 94°C for 4 min followed by 27 cycles of 94°C for 30 s, 52°C for 30 s, 65°C for 1 min, and a final extension for 5 min at 65°C.

RESULTS

Redundant contributions of mediator tail subunits and Srb2p in binding to recombinant Gcn4p in vitro. In previous studies, it was shown that mediator complexes purified from *pgd1*Δ or *gal11*Δ single mutants, which lacked Gal11p, Med2p, and Pgd1p, failed to bind recombinant Gcn4p in vitro (23, 29, 33). As indicated above, it seemed possible that the native mediator complexes present in these mutants might not lack all three subunits of the Gal11p/Med2p/Pgd1p triad. Accordingly, we prepared WCEs from mutants with single deletions of these or other mediator subunits and analyzed the binding of the mutant complexes to recombinant GST-Gcn4p. In addition to the mutants lacking a tail subunit (*gal11*Δ, *pgd1*Δ, *med2*Δ, and *sin4*Δ mutants), we also analyzed three mutants lacking head domain subunits (*srb2*Δ, *srb5*Δ, and *rox3*Δ mutants) which showed defects in activation by Gcn4p in vivo (Gcn^- phenotype) and the *med1*Δ mutant, which showed WT activation by Gcn4p (40). (According to the *Saccharomyces* Genome Database at http://www.yeastgenome.org, it was reported that *ROX3* is essential. The *rox3*Δ strain employed here, produced by the *Saccharomyces* Deletion Project [10], has strong slow-growth and Gcn^- phenotypes, which are complemented by an episomal copy of *ROX3* [40].) Mediator binding to GST-Gcn4p was assayed by Western analysis using antibodies against mediator subunits on fractions bound to glutathione affinity resin. In these assays, we employed the WT GST-Gcn4p fusion and a mutant derivative with 10 alanine substitutions in the hydrophobic clusters of the Gcn4p activation domain (10Ala fusion).

TABLE 1. Strains used in this study

Strain	Relevant genotype	Reference or Source
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	ResGen[a]
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	ResGen
249	*MAT***a** *gcn4Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	ResGen
1742	*MAT***a** *gal11Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	ResGen
1976	*MAT***a** *sin4Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	ResGen
4393	*MAT***a** *pgd1Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	ResGen
11742	*MATα gal11Δ::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	ResGen
11976	*MATα sin4Δ::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	ResGen
13701	*MATα med2Δ::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	ResGen
LS01	*MAT***a** *med2Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	40
4734	*MAT***a** *srb5Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	ResGen
6611	*MAT***a** *srb2Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	ResGen
3119	*MAT***a** *rox3Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	ResGen
5489	*MAT***a** *med1Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	ResGen
LS03	*MATα med2Δ::kanMX4 gal11Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
LS04	*MAT***a** *srb2Δ::kanMX4 gal11Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
LS08	*MAT***a** *srb2Δ::kanMX4 sin4Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 lys2Δ0*	This study
LS09	*MAT***a** *sin4Δ::kanMX4 gal11Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
LS10	*MATα pgd1Δ::kanMX4 gal11Δ::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0*	This study
LS11	*MATα pgd1Δ::kanMX4 sin4Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
LS12	*MAT***a** *med2Δ::kanMX4 sin4Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
LS20	*MATα med2Δ::kanMX4 pgd1Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
LS22	*MATα srb2Δ::kanMX4 srb5Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
LS24	*MAT***a** *srb2Δ::kanMX4 pgd1Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
MSY118	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SRB6-myc$_{13}$::HIS3(S.k.)*[b]	This study
MSY119	*MAT***a** *sin4Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SRB6-myc$_{13}$::HIS3(S.k.)*	This study
FZY254	*MAT***a** *srb2Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SRB6-myc$_{13}$::HIS3(S.k.)*	This study
MSY120	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SRB5-myc$_{13}$::HIS3(S.k.)*	This study
MSY121	*MAT***a** *sin4Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SRB5-myc$_{13}$::HIS3(S.k.)*	This study
FZY232	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SRB6-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.)*[3]	This study
FZY234	*MAT***a** *sin4Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SRB6-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.)*	This study
FZY274	*MAT***a** *gal11Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SRB6-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.)*	This study
FZY276	*MAT***a** *pgd1Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SRB6-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.)*	This study
FZY278	*MAT***a** *med2Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SRB6-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.)*	This study
FZY270	*MAT***a** *srb2Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SRB6-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.)*	This study
FZY122	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL11-myc$_{13}$::HIS3(S.k.)*	This study
FZY124	*MAT***a** *sin4Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL11-myc$_{13}$::HIS3(S.k.)*	This study
FZY152	*MAT***a** *med2Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL11-myc$_{13}$::HIS3(S.k.) lys2Δ0*	This study
FZY156	*MAT***a** *pgd1Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL11-myc$_{13}$::HIS3(S.k.)*	This study
FZY250	*MAT***a** *srb2Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL11-myc$_{13}$::HIS3(S.k.)*	This study
FZY306	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL11-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.)*	This study
FZY308	*MAT***a** *sin4Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL11-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.)*	This study
FZY264	*MAT***a** *srb2Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL11-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.)*	This study
FZY260	*MAT***a** *pgd1Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL11-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.) lys2Δ0*	This study
FZY304	*MAT***a** *med2Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL11-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.) LYS2*	This study
MSY13	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL11-myc$_{13}$::HIS3(S.k.) trp1Δ::hisG(S.t.) MED2-HA$_3$::TRP1*	This study
LS45	*MAT***a** *sin4Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL11-myc$_{13}$::HIS3(S.k.) trp1Δ::hisG(S.t.)MED2-HA$_3$::TRP1*	This study
MSY14	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL11-myc$_{13}$::HIS3(S.k.) trp1Δ::hisG(S.t.) PGD1-HA$_3$::TRP1*	This study
LS47	*MATα sin4Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL11-myc$_{13}$::HIS3(S.k.) trp1Δ::hisG(S.t.) PGD1-HA$_3$::TRP1*	This study
FZY120	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 SIN4-myc$_{13}$::HIS3(S.k.)*	This study
FZY177	*MATα srb2Δ::kanMX4 srb5Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SIN4-myc$_{13}$::HIS3(S.k.)*	This study
FZY167	*MATα srb2Δ::kanMX4 gal11Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SIN4-myc$_{13}$::HIS3(S.k.)*	This study
FZY179	*MATα srb2Δ::kanMX4 pgd1Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SIN4-myc$_{13}$::HIS3(S.k.)*	This study
FZY165	*MATα med2Δ::kanMX4 gal11Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SIN4-myc$_{13}$::HIS3(S.k.)*	This study
FZY169	*MATα pgd1Δ::kanMX4 gal11Δ::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 SIN4-myc$_{13}$::HIS3(S.k.)*	This study
FZY175	*MATα med2Δ::kanMX4 pgd1Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SIN4-myc$_{13}$::HIS3(S.k.)*	This study
LS56	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MED2-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.)*	This study
MSY41	*MATα gal11Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 lys2Δ0 MED2-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.)*	This study
LS57	*MAT***a** *pgd1Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MED2-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.)*	This study
LS58	*MAT***a** *sin4Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MED2-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.)*	This study
FZY401	*MAT***a** *srb2Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MED2-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.)*	This study
FZY217	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 SIN4-myc$_{13}$::HIS3(S.k.) gcn4Δ::hisG(S.t.)*	This study

Continued on following page

TABLE 1—*Continued*

Strain	Relevant genotype	Reference or Source
FZY219	*MAT***a** *gal11*Δ::*kanMX4 his3*Δ*1 leu2*Δ*0 ura3*Δ*0 SIN4-myc*$_{13}$::*HIS3*(*S.k.*) *gcn4*Δ::*hisG*(*S.t.*) *met15*Δ*0 lys2*Δ*0*	This study
FZY223	*MAT***a** *pgd1*Δ::*kanMX4 his3*Δ*1 leu2*Δ*0 ura3*Δ*0 SIN4-myc*$_{13}$::*HIS3*(*S.k.*) *gcn4*Δ::*hisG*(*S.t.*) *met15*Δ*0 lys2*Δ*0*	This study
FZY221	*MAT***a** *med2*Δ::*kanMX4 his3*Δ*1 leu2*Δ*0 ura3*Δ*0 SIN4-myc*$_{13}$::*HIS3*(*S.k.*) *gcn4*Δ::*hisG*(*S.t.*) *met15*Δ*0 lys2*Δ*0*	This study
FZY225	*MAT***a** *srb2*Δ::*kanMX4 his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 SIN4-myc*$_{13}$::*HIS3*(*S.k.*) *gcn4*Δ::*hisG*(*S.t.*)	This study

[a] ResGen, purchased from Research Genetics.
[b] *HIS3*(*S.k.*), the *HIS3* allele from *S. kluyveri.*
[c] *hisG*(*S.t.*), *hisG* sequences from *S. enterica* serovar Typhimurium.

These mutations were shown previously to impair transcriptional activation by Gcn4p in vivo (9, 14, 32).

As expected, all mediator subunits assayed in the WT extract showed substantial binding to WT GST-Gcn4p, greatly reduced binding to the 10Ala mutant protein, and no detectable binding to GST alone (Fig. 1A, WT lanes). The binding of most mediator subunits (considered as a fraction of the input amounts in the extracts) in the *gal11*Δ, *pgd1*Δ, *med2*Δ, *rox3*Δ, and *med1*Δ extracts was similar to that seen in the WT extract; however, a substantial reduction in binding occurred in the *sin4*Δ extract (Fig. 1A). In the *srb2*Δ and *srb5*Δ extracts, only the binding of Srb5p and Srb2p, respectively, was seriously impaired, suggesting that these two subunits are interdependent for stable association with mediator. This is consistent with the fact that Srb2p and Srb5p interact directly with one another in vitro (17). Thus, except for deletion of Sin4p, the deletions of single mediator subunits had limited effects on mediator binding to GST-Gcn4p in vitro.

Consistent with the results from the *sin4*Δ extract, we observed essentially no mediator binding to GST-Gcn4p in the three extracts prepared from double mutants lacking Sin4p and one of the other tail subunits, Gal11p, Pgd1p, or Med2p (Fig. 1B). Importantly, a strong reduction in binding also was observed for the *gal11*Δ *med2*Δ double mutant (Fig. 1B, lanes 17 to 20), and only slightly less severe binding defects occurred with the double mutants lacking Pgd1p and Med2p or Pgd1p and Gal11p (lanes 5 to 8 and 13 to 16). Note that the binding of SAGA and SWI/SNF subunits to GST-Gcn4p occurred at nearly WT levels in all of the double mutants, indicating that the binding defects were specific for mediator subunits. (Although Taf9p and Taf12p are shared by SAGA and TFIID, we showed previously that TFIID does not bind to GST-Gcn4p in these assays (32). It is possible, however, that the binding of Tra1p to GST-Gcn4p reflects interaction of the NuA4 complex, rather than SAGA, with GST-Gcn4p.)

The findings in Fig. 1A and B can be explained by proposing that the *sin4*Δ mutation leads to dissociation of the other three tail subunits from mediator (29), impairing all interactions between Gcn4p and the tail domain. By contrast, the *gal11*Δ, *med2*Δ, and *pgd1*Δ single mutations would leave the other mediator tail subunits intact, and the mutant tail domains lacking only one of these subunits would still interact effectively with GST-Gcn4p. Because the interaction with GST-Gcn4p is lost in all three double mutants lacking two subunits of the Gal11p/Med2p/Pgd1p triad, it is possible that Gcn4p must contact any two of these subunits simultaneously for stable interaction with mediator in vitro. Alternatively, Gcn4p might contact only one of the three subunits, but this interaction requires an indirect contribution from one of the other two subunits of the triad.

Deleting the head domain subunit Srb2p together with the tail subunit Pgd1p, Gal11p, or Sin4p also greatly impaired the binding of mediator to GST-Gcn4p (Fig. 1C and data not shown), comparable to what was observed in the double mutants lacking two tail subunits (Fig. 1B). By contrast, a double deletion of head domain subunits Srb2p and Srb5p had little effect on the binding of the other mediator subunits to GST-Gcn4p (Fig. 1C, lanes 5 to 8). These results suggest that the binding of Srb mediator to Gcn4p involves overlapping contributions from the four tail subunits and the head subunit Srb2p.

It was important to verify that the impaired binding to GST-Gcn4p that occurred with the mutants lacking two mediator subunits did not result from disruption of the remainder of mediator. To this end, we tagged the C terminus of Sin4p with 13 myc epitopes in the mutants described above containing *SIN4*. Extracts from the resulting *SIN4-myc* strains were immunoprecipitated with myc antibodies, and the immune complexes were probed for various mediator subunits. All six mediator subunits we tested from the head or middle domains were immunoprecipitated with myc antibodies from the *SIN4-myc* strain, but not from the untagged *SIN4* strain (Fig. 2A). Importantly, we observed WT association of all head and middle domain subunits with myc-Sin4p in the three double mutants lacking two tail subunits (*gal11*Δ *med2*Δ, *pgd1*Δ *med2*Δ, and *pgd1*Δ *gal11D* mutants) and in the *srb2*Δ *gal11*Δ and *srb2*Δ *pgd1*Δ double mutants (Fig. 2B). Hence, we conclude that the strong defects in binding to GST-Gcn4p observed for these five double mutants (Fig. 1) probably do not result from disruption of the mediator head or middle domains.

Because an otherwise intact mediator complex lacking the tail domain can be purified from a *sin4*Δ strain (8, 29), we expected to find that head and middle domain subunits would also remain associated with one another in the *sin4*Δ extract. To verify this prediction, we myc tagged the head subunit Srb6p in *SIN4* and *sin4*Δ strains and conducted coimmunoprecipitation analysis as described above. As expected, the middle domain subunits Med1p, Rgr1p, and Srb7p and the head subunit Srb2p coimmunoprecipitated with myc-Srb6 in both the *SIN4* and *sin4*Δ strains and also in *srb2*Δ cells (Fig. 2C). We additionally myc tagged Gal11p to confirm that deleting *SIN4* would lead to dissociation of other tail subunits from the head and middle portions of mediator (29). As expected, myc-

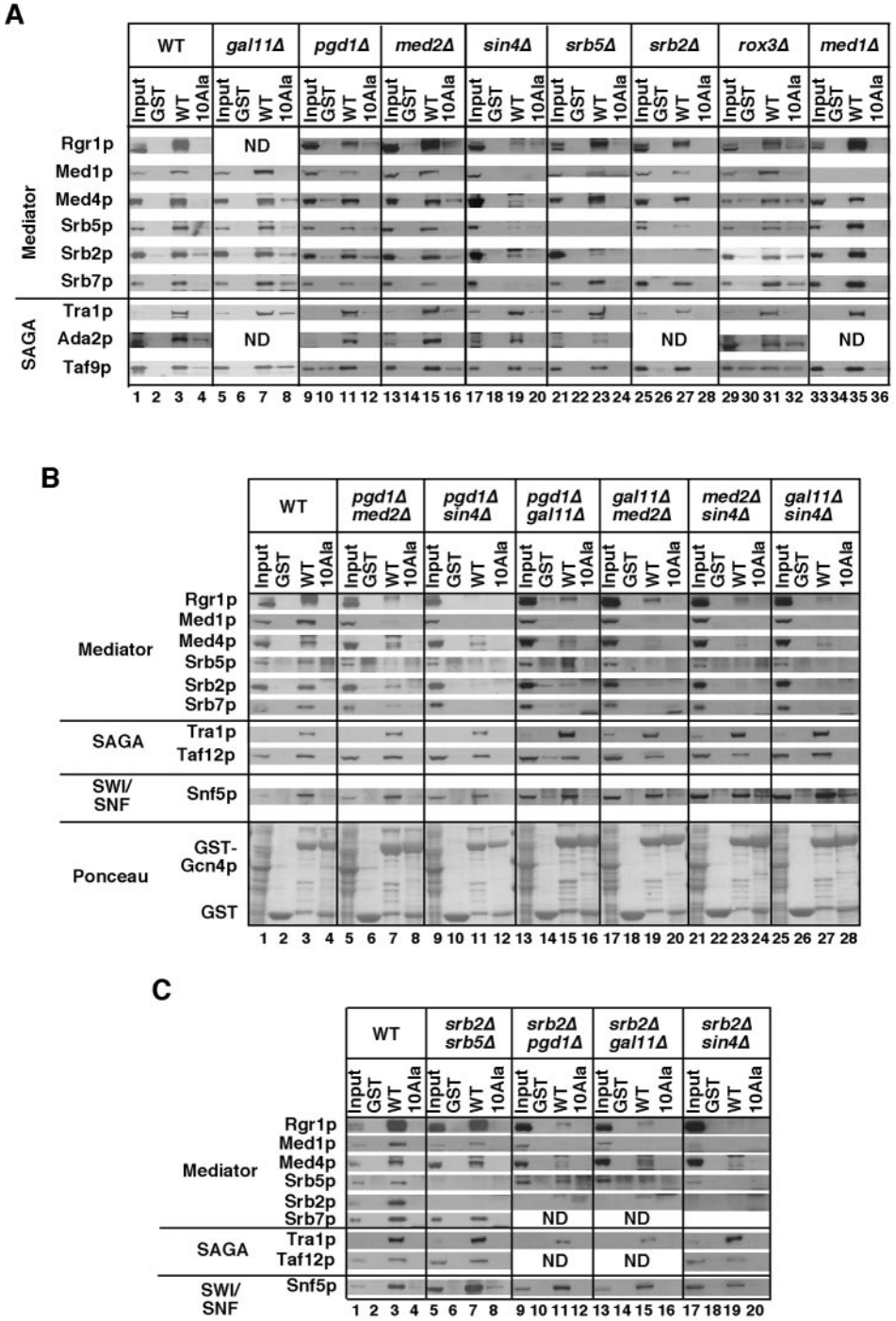

FIG. 1. Overlapping contributions of Srb mediator tail subunits and Srb2p in binding to recombinant GST-Gcn4p in vitro. Equal amounts of GST, WT GST-Gcn4p (WT), and GST-Gcn4-10Ala present in *Escherichia coli* extracts were incubated with 1 mg of the appropriate yeast WCEs and bound to glutathione-Sepharose resin. The bound fractions plus an aliquot of the WCE (input) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subunits of Srb mediator, SAGA, or SWI/SNF listed on the left were detected by Western analysis. The bottom section of panel B shows Ponceau S staining of the Western blots to reveal the amounts of GST proteins precipitated in the assays. (A) Binding assays were conducted using equal amounts of WCEs from yeast strains BY4741 (WT), 1742 (*gal11*Δ), 4393 (*pgd1*Δ), LS01 (*med2*Δ), 1976 (*sin4*Δ), 4734 (*srb5*Δ), 6611 (*srb2*Δ), 3119 (*rox3*Δ), and 5489 (*med1*Δ). (B) Binding assays were conducted using WCEs from strains BY4741 (WT), LS20 (*pgd1*Δ *med2*Δ), LS11 (*pgd1*Δ *sin4*Δ), LS10 (*pgd1*Δ *gal11*Δ), LS03 (*gal11*Δ *med2*Δ), LS12 (*med2*Δ *sin4*Δ), and LS09 (*gal11*Δ *sin4*Δ). (C) Binding assays were conducted using WCEs from strains BY4741 (WT), LS22 (*srb2*Δ *srb5*Δ), LS24 (*srb2*Δ *pgd1*Δ), LS04 (*srb2*Δ *gal11*Δ), and LS08 (*srb2*Δ *sin4*Δ).

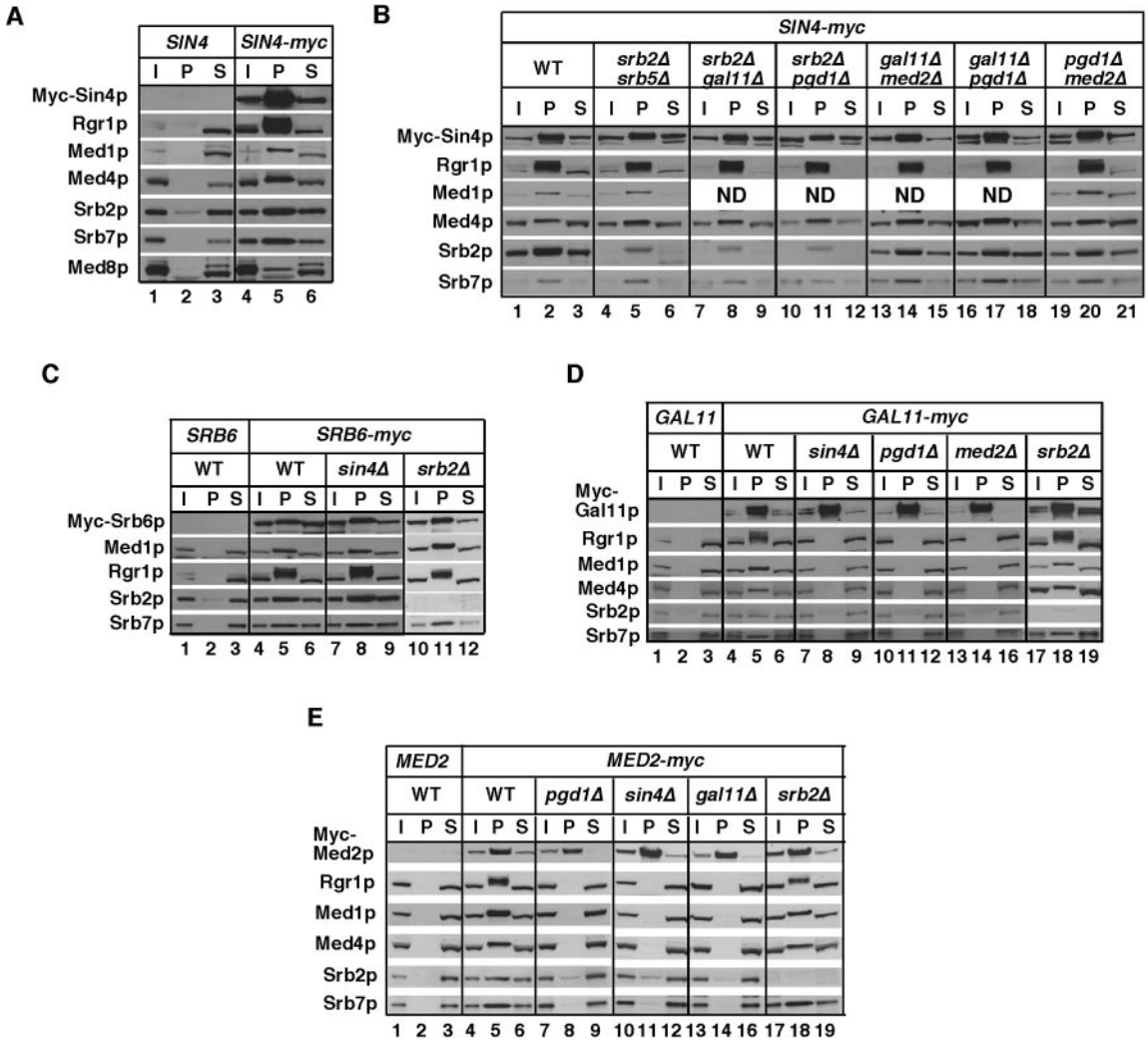

FIG. 2. Coimmunoprecipitation analysis of Srb mediator integrity in mutant strains. WCEs from the appropriate yeast strains were immunoprecipitated with monoclonal c-myc antibodies. The immune complexes were collected, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subjected to Western analysis to detect the proteins listed on the left of each panel or with monoclonal c-myc antibodies to detect the myc-tagged proteins. I, 4% of the input WCE; P, the total pellet fraction from the immunoprecipitate; S, 10% of the supernatant fraction. The bands evident in the immunoprecipitates from the three strains lacking *SRB2* probed with Srb2p antibodies in lanes 5, 8, and 11 of panel B result from nonspecific reactions with the immunoglobulin G light chain that occur variably in these experiments. The following yeast strains were analyzed: BY4741 (*SIN4*) and FZY120 (*SIN4-myc*) (A); *SIN4-myc* strains FZY120 (WT), FZY177 (*srb2*Δ *srb5*Δ), FZY167 (*srb2*Δ *gal11*Δ), FZY179 (*srb2*Δ *pgd1*Δ), FZY165 (*gal11*Δ *med2*Δ), FZY169 (*gal11*Δ *pgd1*Δ), and FZY175 (*pgd1*Δ *med2*Δ) (B); BY4741 (*SRB6 SIN4*), MSY118 (*SRB6-myc SIN4*), MSY119 (*SRB6-myc sin4*Δ), and FZY254 (*SRB6-myc srb2*Δ) (C); BY4741 (*GAL11*; WT), FZY122 (*GAL11-myc*; WT), FZY124 (*GAL11-myc sin4*Δ), FZY156 (*GAL11-myc pgd1*Δ), FZY152 (*GAL11-myc med2*Δ), and FZY250 (*GAL11-myc srb2*Δ) (D); and BY4741 (*MED2*; WT), LS56 (*MED2-myc*; WT), LS57 (*MED2-myc pgd1*Δ), LS58 (*MED2-myc sin4*Δ), MSY41 (*MED2-myc gal11*Δ), and FZY401 (*MED2-myc srb2*Δ) (E).

Gal11p was dissociated from the head and middle domain subunits in the *sin4*Δ extract (Fig. 2D, cf. lanes 4 to 6 and 7 to 9). We also found that myc-Gal11p failed to coimmunoprecipitate with middle and head subunits in extracts lacking the tail subunits Pgd1p or Med2p, but not in the extract lacking head subunit Srb2p (Fig. 2D, lanes 10 to 19 versus 4 to 6). Results essentially identical to those shown for the *GAL11-myc* strains shown in Fig. 2D were obtained with a panel of strains harboring myc-tagged Med2p (Fig. 2E).

The dissociation of myc-Gal11p from the rest of mediator in the *med2*Δ and *pgd1*Δ extracts seems at odds with our finding that binding of mediator to GST-Gcn4p was more severely impaired in extracts of the *gal11*Δ *med2*Δ and *gal11*Δ *pgd1*Δ double mutants than in extracts of the *med2*Δ and *pgd1*Δ single mutants (Fig. 1A and B), which implied that Gal11p was still associated with mediator in the *med2*Δ and *pgd1*Δ single mutants. To explain this discrepancy, we propose that adding the myc_{13} tag to Gal11p weakens its association with other tail subunits and that combining this alteration with the elimination of Pgd1p or Med2p from the tail domain leads to dissociation of myc-tagged Gal11p from the rest of mediator. An analogous explanation would apply to the failure of myc-tagged Med2p to coimmunoprecipitate with other mediator subunits from *gal11*Δ or *pgd1*Δ extracts (Fig. 2E) even though the GST pull-down data in Fig. 1 strongly suggested

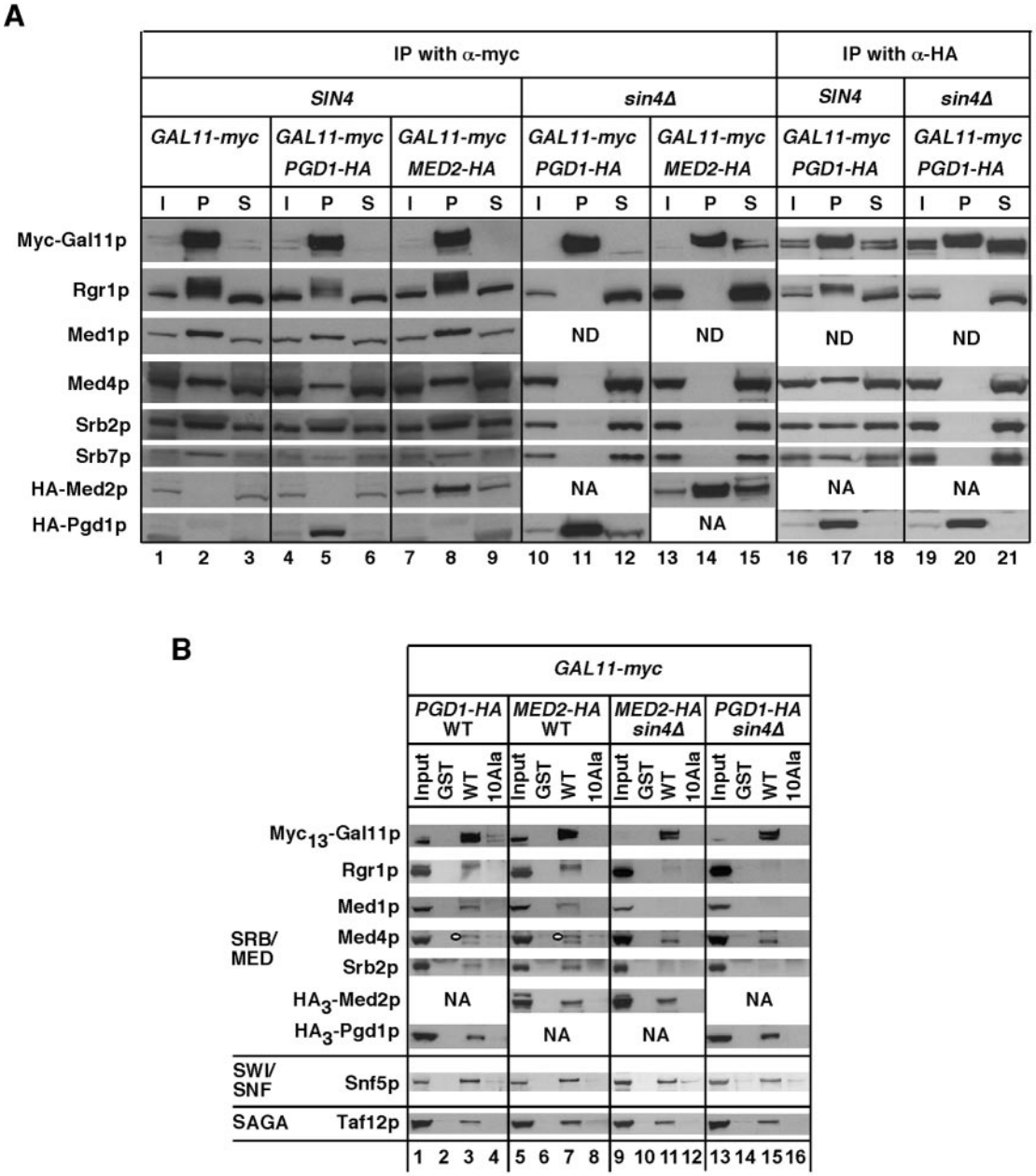

FIG. 3. The Gal11p/Med2p/Pgd1p subcomplex in *sin4*Δ extracts binds to GST-Gcn4p in vitro. (A) WCEs from the appropriate yeast strains were immunoprecipitated (IP) with myc or HA antibodies as indicated. The immune complexes were subjected to Western analysis to detect the proteins listed on the left using c-myc antibodies to detect myc-Gal11p and HA antibodies to detect HA-Med2p and HA-Pgd1p. I, 4% of the input WCE; P, the total pellet fraction from the immunoprecipitate; S, 10% of the supernatant fraction. The following yeast strains were employed: FZY122 (*SIN4 GAL11-myc*), MSY14 (*SIN4 GAL11-myc PGD1-HA*), MSY13 (*SIN4 GAL11-myc MED2-HA*), LS47 (*sin4*Δ *GAL11-myc PGD1-HA*), and LS45 (*sin4*Δ *GAL11-myc MED2-HA*). (B) GST pull-down assays were carried out as described for Fig. 1 using the following yeast strains: MSY14 (*GAL11-myc PGD1-HA SIN4*), MSY13 (*GAL11-myc MED2-HA SIN4*), LS45 (*GAL11-myc MED2-HA sin4*Δ), and LS47 (*GAL11-myc PGD1-HA sin4*Δ). White ovals mark the locations of Med4p in lanes 3 and 7; the bands beneath them in lanes 3, 7, 11, and 15 are cross-reacting species.

that Med2p still resides in the mediator complexes found in *gal11*Δ or *pgd1*Δ extracts.

Gcn4p can bind in vitro to a mediator tail subcomplex present in *sin4*Δ extracts. It was of interest to ascertain whether myc-Gal11p remains associated with Med2p and Pgd1p in a stable subcomplex in *sin4*Δ cells. To determine this, we tagged Med2p and Pgd1p with three tandem HA epitopes in the *sin4*Δ *GAL11-myc* and *SIN4 GAL11-myc* strains and conducted coimmunoprecipitation assays. The mediator head and middle subunits coimmunoprecipitated with myc-Ga111p from the *SIN4* extract, but not from the *sin4*Δ extract (Fig. 3A, lanes 10 to 15 versus 1 to 9), in agreement with the results described above (Fig. 2D). Importantly, HA-Med2p and HA-Pgd1p coimmunoprecipitated with myc-Gal11p at high levels

from both *sin4*Δ and *SIN4* extracts (Fig. 3A, cf. lanes 4 to 9 and 10 to 15). Similarly, when HA antibodies were used, only myc-Gal11p coimmunoprecipitated with HA-Pgd1p from the *sin4*Δ extract, whereas all of the mediator subunits coimmunoprecipitated with HA-Pgd1p from the *SIN4* extract (Fig. 3A, lanes 16 to 18 versus 19 to 21). (The apparent increase in association of HA-Pgd1p and HA-Med2p with myc-Gal11p in the *sin4*Δ strain versus the *SIN4* strain, evident in lanes 5, 8, 11, and 14 of Fig. 3A, was not observed consistently, as seen by comparing lanes 17 and 20 of Fig. 3A.) Based on the results shown in Fig. 3A, we conclude that myc-Gal11p forms a stable subcomplex with HA-Med2p and HA-Pgd1p in vivo in the absence of Sin4p.

We next addressed whether the myc-Gal11p/HA-Med2p/HA-Pgd1p subcomplex can interact with Gcn4p in vitro. As shown in Fig. 3B, myc-Gal11p, HA-Med2p, and HA-Pgd1p all showed strong binding to GST-Gcn4p in *sin4*Δ extracts at levels comparable to that seen in the corresponding *SIN4* extracts. As expected, the binding of five subunits from the head and middle domains was abolished in the *sin4*Δ extracts. These results show that the myc-Gal11p/Pgd1p/Med2p subcomplex in *sin4*Δ extracts can interact specifically with GST-Gcn4p. It could be argued that Gal11p, Med2p, and Pgd1p bind to GST-Gcn4p in the *sin4*Δ extract as components of the Paf1 complex, as Gal11p has been found associated with this alternative mediator complex (39). However, our previous finding that myc-Paf1p in cell extracts does not bind specifically to GST-Gcn4p (40) is at odds with this possibility. Moreover, we found here that myc-Paf1p does not coimmunoprecipitate with HA-Med2p or HA-Pgd1p (data not shown).

Requirements for tail domain subunits and Srb2p in recruitment of the mediator head domain by Gcn4p in vivo. We next asked whether the mediator tail subunits and Srb2p are required for recruitment of mediator by Gcn4p to target promoters in vivo. By ChIP analysis, we showed previously that Gcn4p can recruit myc-tagged mediator subunits Srb6p, Gal11p, and Sin4p to the *ARG1* UAS when Gcn4p synthesis is induced by starvation for isoleucine and valine with the inhibitor SM (40). Hence, we set out to determine the effects of deleting single subunits of Srb mediator on the recruitment of myc-Srb6p to *ARG1* in SM-treated cells.

We first conducted ChIP analysis on six pairs of yeast strains, all harboring the *SRB6-myc* allele, containing either all other WT mediator genes or a single deletion of *SIN4*, *GAL11*, *PGD1*, *MED2*, or *SRB2*. One member of each pair contained *gcn4*Δ while the other carried episomal *GCN4*, and all strains were grown in the presence of SM to induce Gcn4p synthesis in the *GCN4* strains. In agreement with previous results, the *ARG1* UAS was immunoprecipitated with myc-Srb6p at a level approximately fivefold higher in the *GCN4* strains than in the *gcn4*Δ strains containing all WT mediator subunits (Fig. 4A and B, WT lanes). By contrast, we observed low-level recruitment of myc-Srb6p to *ARG1* in all five *GCN4* strains lacking single mediator subunits (Fig. 4A and B). The amount of Gcn4p-dependent binding of myc-Srb6p to the UAS in each mutant was expressed as a percentage of that measured in WT to yield the values listed below the histogram in Fig. 4B. The results show that all five subunit deletions reduced Gcn4p-dependent recruitment of myc-Srb6p at *ARG1* to ≤10% of the WT level. Deleting each tail domain subunit, or Srb2p, also had a marked effect on recruitment of myc-Srb6p by Gcn4p to the *SNZ1* UAS (Fig. 4C).

Western analysis of myc-Srb6p revealed no significant differences in myc-Srb6p levels between the WT and mutant strains analyzed in Fig. 4 (data not shown). Moreover, myc-Srb6p coimmunoprecipitated at similar levels with head and middle domain subunits from extracts of these WT and mutant strains (Fig. 2C and data not shown). Thus, the reductions in myc-Srb6p recruitment in these mutants do not result from disruption of mediator or decreased steady-state levels of myc-Srb6p.

It was also important to determine whether the binding of Gcn4p itself to the target genes was affected by deletions of mediator subunits. To address this possibility, we introduced a plasmid-borne *GCN4-myc* allele into the *gcn4*Δ strains containing mediator subunit deletions (or the otherwise WT *gcn4*Δ strain) and conducted ChIP assays to measure myc-Gcn4p binding to the *ARG1* promoter. All of the mutants displayed myc-Gcn4p binding to the *ARG1* UAS at levels greater than or equal to that seen in the corresponding WT strain (34) (data not shown). Thus, we conclude that the mediator tail subunits and Srb2p are required for recruitment of the Srb mediator head domain by promoter-bound Gcn4p.

Evidence that the tail domain can be recruited independently by Gcn4p in *sin4*Δ cells. Having shown that the Gal11p/Pgd1p/Med2p subcomplex is dissociated from the rest of mediator in the *sin4*Δ extract and that it can interact specifically with GST-Gcn4p in vitro, we conducted ChIP assays to determine whether Gcn4p can recruit this subcomplex to target promoters in *sin4*Δ cells. We began by analyzing strains containing *GAL11-myc* (Fig. 4D). Remarkably, we saw no reduction in recruitment of myc-Gal11p to *ARG1* in the *sin4*Δ strain and only a modest reduction in binding (to ~80% of WT) in the *pgd1*Δ and *srb2*Δ mutants (Fig. 4E). Only the *med2*Δ mutation strongly impaired recruitment of myc-Gal11p to *ARG1*. Similar results were obtained at *SNZ1* (Fig. 4F) except that *sin4*Δ, *pgd1*Δ, and *srb2*Δ had relatively stronger effects on myc-Gal11p binding at this gene. Nevertheless, these three mutations all produced much smaller reductions in recruitment of myc-Gal11p than of myc-Srb6p at *SNZ1* (cf. Fig. 4E and F to B and C). Deletion of *MED2* greatly impaired myc-Gal11p binding at *SNZ1*, as observed at *ARG1*. We verified by Western analysis that the steady-state levels of myc-Gal11p in WT and *med2*Δ extracts are similar (data not shown). The findings in Fig. 4F and G imply that high-level recruitment of myc-Gal11p by Gcn4p is relatively independent of Sin4p and other mediator subunits and may depend only on Med2p.

To obtain additional evidence that the Gal11p/Pgd1p/Med2p triad can be recruited independently of the rest of mediator, we conducted ChIP analysis of strains containing myc-tagged Med2p. Our coimmunoprecipitation analysis had indicated that myc-Med2p was dissociated from the rest of mediator by the *sin4*Δ, *gal11*Δ, or *pgd1*Δ mutation (Fig. 2E). In ChIP analysis, we observed strong Gcn4p-dependent recruitment of myc-Med2p to *ARG1* and *SNZ1* in the *sin4*Δ strain but weak recruitment of myc-Med2p in the *gal11*Δ and *pgd1*Δ mutants (Fig. 5A and B). The identical conclusion emerged from ChIP analysis of strains containing *GCN4* on a single-copy versus multicopy plasmid, the only difference being that Gcn4p-dependent recruitment was uniformly lower in the

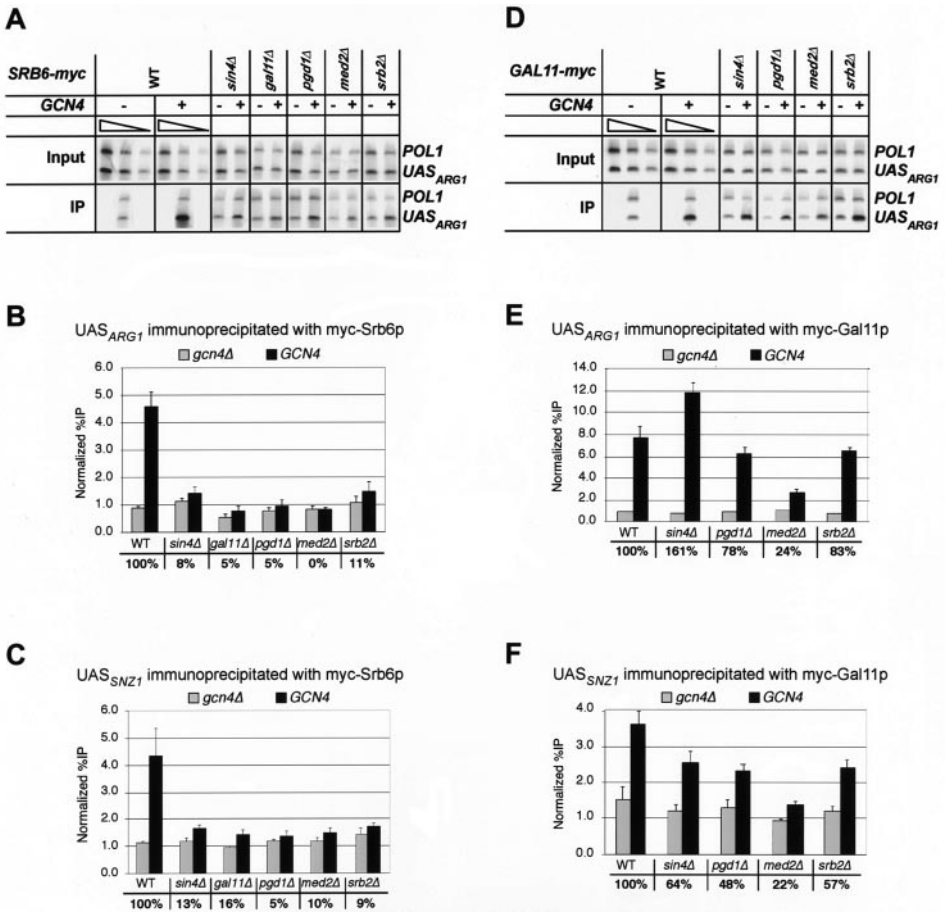

FIG. 4. ChIP analysis of myc-Srb6p and myc-Gal11p recruitment by Gcn4p in mutants lacking different Srb mediator subunits. (A and D) *gcn4*Δ strains expressing myc-Srb6p (A) or myc-Gal11p (D) and containing the indicated mediator subunit deletions were transformed with high-copy-number *GCN4* plasmid pHQ1239 (+ lanes) or empty vector YEplac195 (− lanes), grown for 3 h in SC lacking Ura, Arg, and Ilv and containing 0.5 μg of SM/ml, and subjected to ChIP analysis using antimyc antibodies. The immunoprecipitated DNA containing the *ARG1* UAS or *POL1* ORF (IP) and the corresponding input amounts (input) were measured by quantitative PCR. Three different amounts of input samples were analyzed for the *GCN4* and *gcn4*Δ strains with WT mediator subunits, shown in the first six lanes, to demonstrate the linear response of the PCR signals to the amounts of input DNA. (B and E) The PCR products from the experiments described for panels A and D, respectively, were quantified by phosphorimaging analysis, and the ratios of signals in the IP to the input samples for the *ARG1* UAS were normalized for the corresponding ratios for *POL1* (normalized %IP). The resulting values from three PCR amplifications of chromatin immunoprecipitated from two independent cultures analyzed for each *SRB6-myc* (B) or *GAL11-myc* (E) strain were averaged, and the mean values and standard errors were plotted in the histograms. The percentages below the histograms give the proportions of the WT Gcn4p-dependent binding of the myc-tagged protein to the *ARG1* UAS, calculated by subtracting the normalized ratios for the *gcn4*Δ strain from the normalized ratios for the *GCN4* strain for that mutant and dividing by the corresponding value obtained for the WT pair of *GCN4* and *gcn4*Δ strains. (C and F) The procedures described for panels B and E were employed to analyze binding of myc-Srb6p (C) or myc-Gal11p (F) to the *SNZ1* UAS. The following yeast strains were employed in panels A to C: FZY232 (*gcn4*Δ *SRB6-myc*), FZY234 (*gcn4*Δ *SRB6-myc sin4*Δ), FZY274 (*gcn4*Δ *SRB6-myc gal11*Δ), FZY276 (*gcn4*Δ *SRB6-myc pgd1*Δ), FZY278 (*gcn4*Δ *SRB6-myc med2*Δ), and FZY270 (*gcn4*Δ *SRB6-myc srb2*Δ). The strains analyzed in panels D to F were FZY306 (*gcn4*Δ *GAL11-myc*), FZY308 (*gcn4*Δ *GAL11-myc sin4*Δ), FZY260 (*gcn4*Δ *GAL11-myc pgd1*Δ), FZY304 (*gcn4*Δ *GAL11-myc med2*Δ), and FZY264 (*gcn4*Δ *GAL11-myc srb2*Δ).

strains harboring single-copy *GCN4* (Fig. 5A). (Using a *GCN4-myc* allele, we showed that the binding of myc-Gcn4p at *ARG1* is approximately twofold higher when myc-Gcn4p is expressed from a multicopy versus single-copy plasmid [34].) The data in Fig. 5A and B indicate that Med2p can be recruited efficiently in the *sin4*Δ mutant as a component of the isolated tail domain, dependent on Gal11p and Pgd1p. The fact that recruitment of myc-Med2p shows a greater requirement for Pgd1p than does recruitment of myc-Gal11p may be explained by our observation that myc-Med2p levels are generally lower in the *pgd1*Δ strain than in WT and *gal11*Δ strains (Fig. 2E and data not shown).

The results above showed that recruitment of the tail subunits myc-Gal11p and myc-Med2p exhibits little requirement for Sin4p whereas recruitment of head subunit Srb6p is strongly dependent on Sin4p. We extended this distinction to a second head subunit by conducting ChIP assays on *SIN4* and *sin4*Δ strains containing myc-tagged Srb5p. As shown in Fig. 5C, recruitment of myc-Srb5p to *ARG1* was greatly impaired by the *sin4*Δ mutation, just as we observed for myc-Srb6p (Fig. 4B). This supports the idea that recruitment of the head domain is strongly dependent on its association with the tail domain of mediator.

The coimmunoprecipitation analysis of the *SIN4-myc* strains

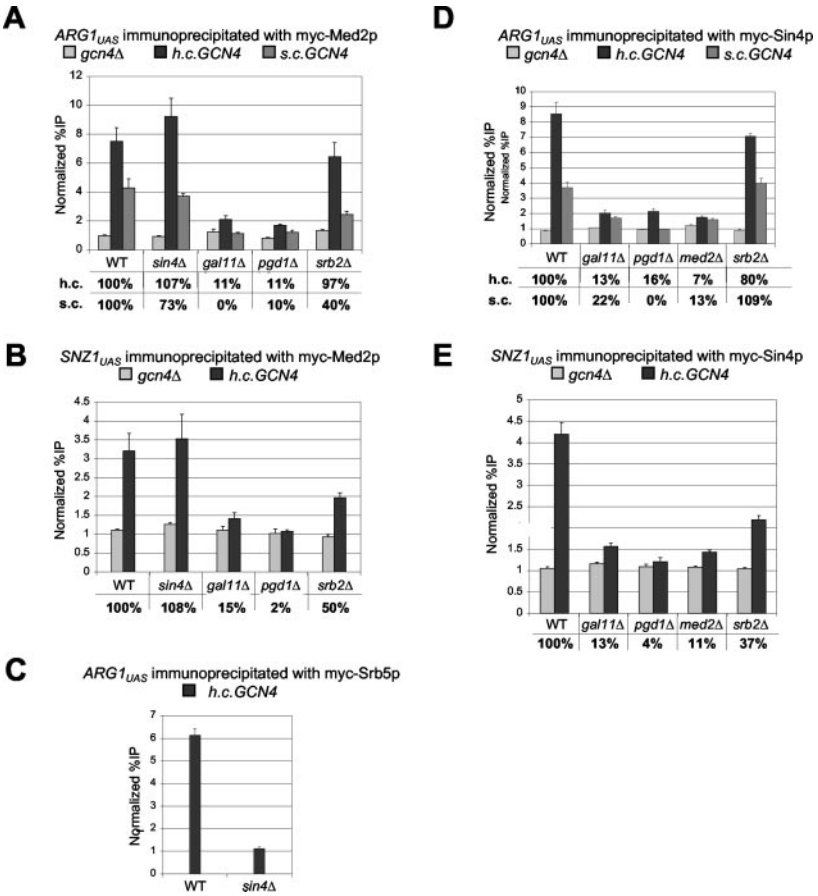

FIG. 5. ChIP analysis of myc-Med2p, myc-Srb5p, and myc-Sin4p recruitment by Gcn4p in mutants lacking different Srb mediator subunits. ChIP analysis was conducted as described in Fig. 4 using *gcn4*Δ strains transformed with either empty vector YEplac195 (*gcn4*Δ), high-copy-number *GCN4* plasmid pHQ1239 (*h.c.GCN4*), or single-copy *GCN4* plasmid p2382 (*s.c.GCN4*). The following strains were employed: LS56 (*gcn4*Δ *MED2-myc*), LS58 (*gcn4*Δ *MED2-myc sin4*Δ), MSY41 (*gcn4*Δ *MED2-myc gal11*Δ), LS57 (*gcn4*Δ *MED2-myc pgd1*Δ), and FZY401 (*gcn4*Δ *MED2-myc srb2*Δ) (A and B); MSY120 (*SRB5-myc*) and MSY121 (*SRB5-myc sin4*Δ) (C); and FZY217 (*gcn4*Δ *SIN4-myc*), FZY219 (*gcn4*Δ *SIN4-myc gal11*Δ), FZY223 (*gcn4*Δ *SIN4-myc pgd1*Δ), FZY221 (*gcn4*Δ *SIN4-myc med2*Δ), and FZY225 (*gcn4*Δ *SIN4-myc srb2*Δ) (D and E).

described above had indicated that myc-Sin4p remained associated with the rest of mediator in the presence of the *med2*Δ, *gal11*Δ, *pgd1*Δ, or *srb2*Δ mutation (Fig. 2B). Hence, we expected to find that recruitment of myc-Sin4p by Gcn4p in vivo would have the same subunit requirements observed for myc-Srb6p. In accordance with this prediction, recruitment of myc-Sin4p to *ARG1* was strongly impaired by the *med2*Δ, *gal11*Δ, and *pgd1*Δ mutations, whether *GCN4* was present on single- or multicopy plasmids (Fig. 5D). Interestingly, Srb2p appears to be dispensable for myc-Sin4p binding at *ARG1* but is required for high-level myc-Sin4p recruitment at *SNZ1* (Fig. 5E). The last findings suggest that Srb2p may be less critical than the tail subunits for Srb mediator recruitment by Gcn4p. In addition, there appears to be more-stringent requirements for mediator recruitment at *SNZ1* than at *ARG1*, similar to what we observed previously for recruitment of SWI/SNF by Gcn4p at these two genes (43).

Effects of deleting mediator subunits on transcriptional activation by Gcn4p in vivo. Having concluded that deletions of each of the four tail domain subunits impaired the recruitment of myc-Srb6p, we compared the effects of these deletions on transcriptional activation by Gcn4p. We showed previously that the *gal11*Δ, *med2*Δ, and *pgd1*Δ strains show increased sensitivity to SM, consistent with impaired transcriptional activation of the *ILV2* gene by Gcn4p. They also were defective for induction of a *lacZ* reporter gene containing tandem Gcn4p binding sites upstream of the *CYC1* promoter, showing induction levels of only ~30% of WT. However, the *sin4*Δ mutant showed nearly WT resistance to SM and was not defective for induction of the Gcn4p-dependent *lacZ* reporter (40).

In the present study, we conducted Northern analysis of *ARG1* and *SNZ1* mRNAs using the same inducing conditions employed for ChIP assays (Fig. 6A). The levels of transcripts were quantified from two or more independent cultures and the mean values were plotted as a fraction of the WT value in the histogram shown in Fig. 6A (see legend for details). Among the single mutants, only the *med2*Δ strain showed strong reductions in the mRNA levels for both genes, decreasing induction of *ARG1* and *SNZ1* mRNAs by ~90 and 70%, respectively (Fig. 6A). The *pgd1*Δ single mutation reduced the induction of *ARG1* and *SNZ1* mRNAs by 20 and 40%, respectively, whereas the *gal11*Δ mutation decreased *ARG1* mRNA by 30% but did not impair induction of *SNZ1* mRNA. Consis-

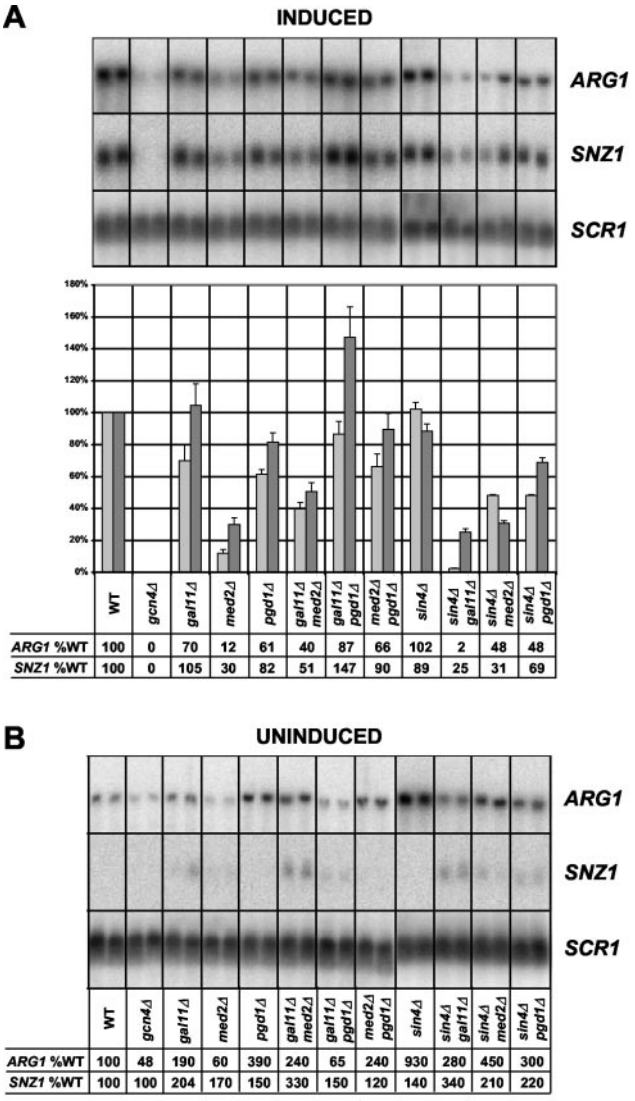

FIG. 6. Northern analysis of *ARG1* and *SNZ1* mRNA expression in mutants lacking single or double tail domain subunits. (A) Total RNA was isolated from strains grown under the inducing conditions described for Fig. 4 and subjected to Northern analysis, probing for *ARG1* and *SNZ1* mRNAs and for *SCR1* RNA as a loading control. Adjacent lanes derive from duplicate cultures of the same strains. The *ARG1* and *SNZ1* signals were quantified with a phosphorimager and normalized for the cognate *SCR1* signals. The normalized average for the *gcn4*Δ strain was subtracted from the normalized averages for all other strains, and the differences were plotted in the histogram as the percentages of the corresponding difference obtained for WT. The light and dark bars for each strain indicate the results obtained from duplicate cultures, as described above. (B) Percentages of WT levels of *ARG1* and *SNZ1* mRNAs in mediator mutants grown under noninducing conditions. The analysis for panel A was carried out on the same strains grown in the absence of SM. The Northern signals for each mutant were expressed as percentages of the corresponding value for WT and are listed below the mutant genotype. The following strains were analyzed: BY4741 (*GCN4*), 249 (*gcn4*Δ), 1742 (*GCN4 gal11*Δ), LS01 (*GCN4 med2*Δ), 4393 (*GCN4 pgd1*Δ), LS03 (*GCN4 gal11*Δ *med2*Δ), LS10 (*GCN4 gal11*Δ *pgd1*Δ), LS20 (*GCN4 med2*Δ *pgd1*Δ), 1976 (*GCN4 sin4*Δ), LS09 (*GCN4 sin4*Δ *gal11*Δ), LS12 (*GCN4 sin4*Δ *med2*Δ), and LS11 (*GCN4 sin4*Δ *pgd1*Δ).

tent with our previous findings, *sin4*Δ had little or no effect on the induction of either transcript. Thus, Med2p seems to play the most critical role, while Sin4p is dispensable for high-level transcription of these genes under inducing conditions.

Interestingly, the *pgd1*Δ and *sin4*Δ single mutants showed a marked derepression (approximately ninefold and fourfold, respectively) of *ARG1* mRNA under noninducing conditions (Fig. 6B). In fact, *sin4*Δ cells showed nearly the same levels of *ARG1* mRNA under noninducing and inducing conditions (cf. Fig. 6A and B). Consistent with this, we found previously that *sin4*Δ produced a fivefold derepression of the Gcn4p-dependent *CYC1-lacZ* reporter described above and that *pgd1*Δ produced an approximately fivefold derepression of a *HIS3-GUS* reporter under noninducing conditions (40). The *gal11*Δ mutation also conferred a moderate (approximately twofold) derepression of both *ARG1* and *SNZ1* mRNAs in the uninduced cells (Figs. 6B). Thus, it seems that inactivation of Sin4p, Pgd1p, or Gal11p derepresses promoter activity at low levels of Gcn4p binding to the UAS under noninducing conditions, even though Pgd1p and Gal11p are required for optimal transcriptional activation by Gcn4p under inducing conditions.

We also conducted Northern analysis on double mutants lacking two different subunits of the tail domain. All of the double mutants involving *med2*Δ had less-severe induction defects than did the *med2*Δ single mutant (Fig. 6A). Thus, *gal11*Δ, *pgd1*Δ, and *sin4*Δ partially overcome the activation defect conferred by *med2*Δ, possibly through their derepressing effects on promoter function. On the other hand, *sin4*Δ exacerbated the modest activation defects conferred by the *pgd1*Δ and *gal11*Δ mutations (Fig. 6A). The *sin4*Δ *gal11*Δ double mutant also showed a synthetic slow-growth phenotype on nonstarvation medium (data not shown). The last findings suggest that there are additive defects in recruitment or coactivator functions of mediator produced by simultaneously deleting Sin4p and Pgd1p or Gal11p which outweigh the derepression of promoter activity produced by eliminating these tail subunits individually.

Med2p, but not Sin4p, is required for promoter binding by TBP and Pol II occupancy in the coding region at *ARG1*. We next compared levels of recruitment of TBP and Pol II by Gcn4p in the *med2*Δ and *sin4*Δ mutants. ChIP analysis with antibodies against TBP or the Pol II subunit Rpb1p showed an approximately threefold-higher level of binding by TBP and Rpb1p to the *ARG1* promoter and an approximately threefold greater association of Rpb1p with the *ARG1* ORF in the WT strains containing episomal *GCN4* than in those carrying *gcn4*Δ (Fig. 7, WT bars). These results demonstrate that Gcn4p recruits TBP and Pol II to the promoter in the course of stimulating *ARG1* transcription, as also shown recently (34). In the *med2*Δ strain, we observed strong reductions in recruitment of TBP and Rpb1p to the promoter and decreased Rpb1p occupancy in the *ARG1* ORF (Fig. 7), all consistent with the impaired induction of *ARG1* mRNA seen in this mutant (Fig. 6A). Thus, Med2p is required for the stimulation of PIC assembly by Gcn4p at *ARG1*. In accordance with the WT induced level of *ARG1* mRNA in the *sin4*Δ strain (Fig. 6A), we observed only a small reduction in TBP recruitment to the promoter (Fig. 7A) and no reduction in Rpb1p association with the *ARG1* ORF (Fig. 7C) in this mutant. Thus, it appears

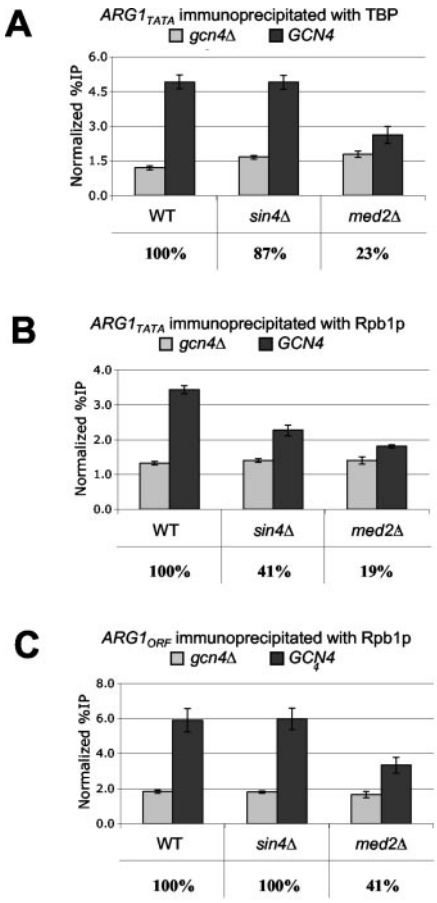

FIG. 7. ChIP analysis of Gcn4p-dependent binding of TBP and Rpb1p to the *ARG1* promoter and coding sequences in *med2*Δ and *sin4*Δ mutants. The following *gcn4*Δ strains were transformed with high-copy-number plasmid pHG1239 (*GCN4*) or empty vector (*gcn4*): FZY232 (*gcn4*Δ *SRB6-myc*), FZY234 (*gcn4*Δ *SRB6-myc sin4*Δ), and FZY278 (*gcn4*Δ *SRB6-myc med2*Δ). ChIP analysis was done as described for Fig. 4 except that antibodies against TBP or Rpb1p were used for the immunoprecipitations and primers to amplify the *ARG1* TATA element (A and B) or 3′ end of the *ARG1* ORF (C) were included.

that deletion of Sin4p and the attendant dissociation of the tail domain from the rest of mediator have little impact on the ability of mediator to promote TBP recruitment and transcription initiation. Considering that the Gal11p/Med2p/Pgd1p triad is the predominant entity containing Med2p that is recruited to *ARG1* in *sin4*Δ cells (Fig. 4 and 5), these findings could indicate that this triad can carry out Med2p-dependent stimulation of TBP recruitment independently of the rest of Srb mediator. This interesting possibility is discussed further below. We will also address the fact that Pol II occupancy at the promoter, but not in the coding region, of *ARG1* was reduced by the *sin4*Δ mutation (cf. Fig. 7B and C).

DISCUSSION

Multiple tail domain subunits and Srb2p contribute to interaction of mediator with Gcn4p in vitro and in vivo. The results of our in vitro experiments indicate that the tail subunits Gal11p, Pgd1p, and Med2p and the head subunit Srb2p make overlapping contributions to the binding of Srb mediator in cell extracts to recombinant Gcn4p. Whereas single deletions of tail subunits Gal11p, Pgd1p, and Med2p had little effect on mediator binding to GST-Gcn4p, a strong reduction in binding occurred when any pair of these subunits was deleted simultaneously. Consistent with this, deletion of *SIN4* alone nearly abolished the binding of mediator to GST-Gcn4p in vitro (Fig. 1). It was shown previously (29) and confirmed here by coimmunoprecipitation analysis that the mediator complex in *sin4*Δ extracts lacks Gal11p, Pgd1p, and Med2p in addition to Sin4p, accounting for its negligible interaction with GST-Gcn4p. Mediator binding to GST-Gcn4p also was strongly impaired when either Gal11p, Pgd1p, or Med2p was deleted together with head subunit Srb2p. Coimmunoprecipitation experiments showed that the head and middle domains of mediator were still associated with Sin4p in all three of these double mutants lacking Srb2p (Fig. 2B). Hence, the simplest explanation for our in vitro binding data is that Gal11p, Pgd1p, Med2p, and Srb2p make independent contributions to the interaction of mediator with Gcn4p, none of which is uniquely required for stable association between mediator and GST-Gcn4p in vitro. The interaction is severely impaired, however, when the contributions of any two of these four subunits are eliminated simultaneously.

Others have shown that recombinant Gcn4p cannot bind to mediator complexes purified from *gal11*Δ, *med2*Δ, or *pgd1*Δ single mutants. Our results do not contradict these previous findings because the purified complexes in those studies lacked all three subunits, Gal11p, Med2p, and Pgd1p (23, 33), and thus were equivalent to the mediator complex present in our *sin4*Δ extracts. Park et al. reported that GST-Gcn4p binds to recombinant forms of Pgd1p/Hrs1p and Gal11p, but not to Med2p (33). It is possible that deleting Med2p together with Pgd1p or Gal11p leads to loss of the third member of this triad from the mediator. This could explain our finding that eliminating Med2p exacerbates the binding defects caused by removing Gal11p or Pgd1p without having to invoke a direct contact between Med2p and Gcn4p.

Our coimmunoprecipitation analysis showed that myc-Gal11p remains associated with HA-Pgd1p and HA-Med2p in a *sin4*Δ extract in which these subunits are dissociated from the rest of mediator (Fig. 3A). Although interaction of head and middle domain subunits with GST-Gcn4p was impaired by *sin4*Δ, the binding of myc-Gal11p, HA-Pgd1p, and HA-Med2p to GST-Gcn4p was retained in *sin4*Δ extracts (Fig. 3B). These findings indicate that the stable myc-Gal11p/Med2p/Pgd1p triad present in *sin4*Δ cells can interact effectively with recombinant Gcn4p in vitro.

In view of the last results, it might be expected that Sin4p would be the only subunit essential for recruitment of mediator head and middle domains by Gcn4p in vivo, since the other three tail subunits and Srb2p appeared to make redundant contacts with GST-Gcn4p in vitro. However, our ChIP analysis showed that single deletions of each tail subunit, or of Srb2p, strongly impaired recruitment of the head subunit myc-Srb6p by Gcn4p to *ARG1* and *SNZ1* (Fig. 4A to C). Similar results were obtained for recruitment of myc-Sin4p, because deleting any of the other three tail subunits greatly reduced its recruitment to both target genes (Fig. 5D and E). To reconcile these in vivo findings with our in vitro binding data, it could be

proposed that loss of a contact between Gcn4p and mediator resulting from single deletions of *GAL11*, *MED2*, *PGD1*, or *SRB2* can be overcome in vitro by mass action at the high concentrations of recombinant GST-Gcn4p used in the binding assays. The concentration of Gcn4p may be lower in living cells, given the short half-life of Gcn4p in vivo (19). In addition, Gcn4p must compete with other transcriptional activators for mediator binding, and the concentrations of these competing proteins will be far lower than that of recombinant GST-Gcn4p in the in vitro assays.

Srb2p was less critical than the tail subunits for recruitment of myc-Sin4p to *SNZ1*, and it was nearly dispensable for myc-Sin4p recruitment to *ARG1* (Fig. 5D and E). To explain the fact that deletion of Srb2p greatly reduced recruitment of myc-Srb6p (Fig. 4A to C) but had relatively little effect on myc-Sin4p recruitment, it could be proposed that myc tagging Srb6p and deleting Srb2p have additive effects on the interaction of Gcn4p with the head domain in which both of these subunits reside. The compound effects on the head domain produced by combining *srb2*Δ with *SRB6-myc* would be equivalent to a single deletion of *GAL11*, *MED2*, or *PGD1* in weakening the interaction of Srb mediator with Gcn4p in vivo. By contrast, the head domain alteration produced by *srb2*Δ in the *SIN4-myc* strain would be insufficient to reduce mediator recruitment to *ARG1*. By this model, Srb2p is less critical than the tail subunits for recruitment of WT mediator by Gcn4p in vivo.

The Gal11p/Med2p/Pgd1p tail subcomplex can be recruited by Gcn4p independently of the rest of mediator in vivo. Whereas recruitment of head subunits Srb6p and Srb5p by Gcn4p was strongly dependent on Sin4p (Fig. 4A to C and 5C), recruitment of the tail subunits myc-Gal11p and Med2p occurred at high levels in *sin4*Δ cells (Fig. 4D to F and 5A and B). This is the expected outcome if a large fraction of the Gal11p/Med2p/Pgd1p triad is dissociated from the head and middle domains in vivo, as we saw in vitro (Fig. 3A), and if the Gal11p/Med2p/Pgd1p triad can be recruited by Gcn4p independently of the rest of mediator. Thus, these results provide the strongest evidence to date that the mediator tail subunits are direct targets of the Gcn4p activation domain in vivo. The binding of myc-Gal11p to *ARG1* and *SNZ1* was impaired by *med2*Δ (Fig. 4D to F), indicating that myc-Gal11p is dependent on its association with Med2p for efficient recruitment by Gcn4p. Because *pgd1*Δ produced a smaller reduction in myc-Gal11p recruitment, it seems that myc-Gal11p is less dependent on Pgd1p than on Med2p for interaction with Gcn4p. On the other hand, *pgd1*Δ strongly impaired myc-Med2p recruitment at both genes (Fig. 5A and B). That recruitment of myc-Med2p seems to show a greater requirement for Pgd1p than does recruitment of myc-Gal11p may be explained by the fact that myc-Med2p levels are generally lower in the *pgd1*Δ strain than in WT or *gal11*Δ strains (Fig. 2E and data not shown). In any event, it is clear that optimal recruitment of the Gal11p/Med2p/Pgd1p triad by Gcn4p in vivo requires the integrity of its constituent subunits. We propose that complex formation by these proteins produces an extended binding surface that accommodates simultaneous interactions with multiple hydrophobic clusters in the Gcn4p activation domain.

Considering a previous report that Gal11p is associated with the Paf1 complex (39), it could be proposed that the Gal11p/Med2p/Pgd1p triad is recruited in *sin4*Δ cells in the context of the Paf1 complex. We believe that this is unlikely for several reasons. First, we previously detected no binding of Paf1 to GST-Gcn4p in vitro under conditions where the binding of Srb mediator was robust (40). Second, we detected no coimmunoprecipitation of HA-Med2p or HA-Pgd1p with myc-tagged Paf1p from cell extracts under conditions where association of HA-Med2p and HA-Pgd1p with Srb mediator subunits occurred at high levels (Fig. 3A and data not shown). Third, there has been no previous report that Med2p and Pgd1p are associated with the Paf1 complex. Fourth, recent affinity purifications of the Paf1 complex have not identified Gal11p as a subunit, suggesting that it is a very minor constituent of the complex. Fifth, we found recently that deletion of the Cdc73p subunit of the Paf1 complex does not affect recruitment of TBP by Gcn4p to *ARG1* (34), whereas deletion of Med2p clearly does (Fig. 7A). Thus, the function of Med2p in TBP recruitment is not dependent on the intact Paf1 complex. Admittedly, we cannot yet eliminate the possibility that Med2p must function in TBP recruitment in association with the Paf1 complex in *sin4*Δ cells where the association of Med2p with Srb mediator is disrupted.

It is interesting that deleting the head subunit gene *SRB2* generally had a greater effect on recruitment of the tail subunits Gal11p and Med2p than did *sin4*Δ (Fig. 4A to C and 5A and B). This result was unexpected because *sin4*Δ dissociates the Gal11p/Med2p/Pgd1p triad from the rest of mediator, whereas *srb2*Δ has no effect on the association of tail subunits with the rest of mediator (Fig. 2B, D, and E). Thus, it is very unlikely that *srb2*Δ impairs the interaction of tail subunits with one another. To account for the greater effect of *srb2*Δ than of *sin4*Δ on recruitment of Gal11p and Med2p, we suggest that dissociation of the Gal11p/Med2p/Pgd1p subcomplex from the rest of mediator in the *sin4*Δ mutant enhances recruitment of the isolated triad by Gcn4p, compensating for the loss of interaction between Gcn4p and Srb2p in the head domain. Perhaps incorporation of the Gal11p/Med2p/Pgd1p subcomplex into mediator occludes one of its contacts with Gcn4p and necessitates an additional interaction with the head domain via Srb2p. Indeed, single-particle electron microscopy analysis revealed physical interactions between the tail and middle domains of mediator (7) which might obscure an activator binding surface in the tail.

Evidence that the Gal11p/Med2p/Pgd1p triad has a coactivator function in TBP recruitment. Deleting any of the four tail subunits of mediator strongly reduced recruitment of the head and middle domains of Srb mediator by Gcn4p to *ARG1* and *SNZ1*. Because mediator is essential for most Pol II transcription (42), this might be predicted to produce a severe defect in transcriptional activation. However, only the *med2*Δ strain displayed strong reductions in the induced levels of *ARG1* and *SNZ1* mRNAs (Fig. 6A). Our ChIP analysis showed that recruitment of TBP and Pol II to the *ARG1* promoter was greatly impaired in *med2*Δ cells (Fig. 7A and B), accounting for the reduced occupancy of Pol II in the ORF (Fig. 7C) and for the defective induction of *ARG1* mRNA seen in this strain. Thus, Med2p is critically required for the PIC assembly stimulated by Gcn4p, in accordance with our other recent findings (34). Remarkably, *sin4*Δ had little or no effect on TBP recruitment to the promoter (Fig. 7A), even though deletion of Sin4p

eliminates a critical connection between the tail subunits and the rest of mediator. Considering that the Gal11p/Med2p/Pgd1p triad is the predominant mediator entity recruited to *ARG1* in *sin4*Δ cells, it seems possible that Med2p can carry out its function in TBP recruitment in the context of the Gal11p/Med2p/Pgd1p triad independently of the rest of mediator. However, there are some caveats to this conclusion.

It seems improbable that the Gal11p/Med2p/Pgd1p triad alone could support a WT level of TBP recruitment, considering that an *srb4* Ts^- mutation was shown to abolish TBP recruitment by Gal4p (20, 24), plus our finding that the mediator head subunit Rox3p is critical for TBP recruitment by Gcn4p (34). Thus, there are likely to be contributions from both head and tail domains to the TBP recruitment function of mediator. In fact, we always observed a low but significant recruitment of mediator head subunits in the *sin4*Δ strain (Fig. 4B and C). This could result from inefficient recruitment of tailless mediator or from recruitment of a small fraction of otherwise intact mediator lacking only Sin4p which was too unstable to be detected in our coimmunoprecipitation analysis of the *SRB6-myc sin4*Δ strain (Fig. 2C). Because we don't know how much mediator is required at the promoter to support a WT level of TBP recruitment, a small fraction of tailless or Sin4p-less mediator could make a considerable contribution to this function. Hence, it is unclear how much of the TBP recruitment that occurs in *sin4*Δ cells can be attributed to the free Gal11p/Med2p/Pgd1p triad. Nevertheless, it is intriguing that a recent model for the structure of a Pol II/TFIIF/TFIIB/TBP/DNA initiation complex and its interaction with mediator seems to position the tail domain of mediator in the vicinity of TFIIB and TBP (3, 5), consistent with a role in stabilizing TBP binding to the promoter.

Although the *sin4*Δ mutant exhibits a nearly WT level of TBP recruitment, it shows reduced occupancy of Pol II at the *ARG1* promoter. Interestingly, this apparent defect in Pol II recruitment is not associated with smaller amounts of Pol II in the *ARG1* ORF (Fig. 7C). To reconcile these findings, it could be proposed that inactivation of Sin4p increases the rate of promoter clearance and thereby compensates for a reduced rate of Pol II recruitment in the *sin4*Δ mutant. This could yield a net reduction in steady-state promoter occupancy by Pol II without significantly decreasing the transcription rate at *ARG1*. A tacit assumption of this explanation is that promoter clearance is a relatively slow step in WT cells. However, we cannot dismiss the possibility that a conformational change in the PIC associated with *sin4*Δ decreases the efficiency of Pol II crosslinking or immunoprecipitation and does not produce a genuine reduction in Pol II binding to the promoter.

How can we account for the weak phenotypes of *gal11*Δ and *pgd1*Δ mutants given that these two mutations (unlike *sin4*Δ) decrease the recruitment of both head and tail domain subunits by Gcn4p? One possibility is that Gcn4p normally recruits the mediator in excess of the amount needed to support WT transcription, so that the large reductions in mediator recruitment observed in the *gal11*Δ and *pgd1*Δ mutants do not produce a strong decrease in the transcription rate. Physiologically relevant levels of coactivator recruitment may be below the detection limit of the ChIP assay. Gcn4p recruits an array of other coactivators, some of which (SAGA and SWI/SNF) appear to have functions redundant with those of Srb mediator (37). Hence, certain mediator functions may be carried out by other coactivators when mediator recruitment is diminished (but not abolished) by deletion of Gal11p, Pgd1p, or Sin4p. To account for the stronger activation defect seen in *med2*Δ cells, we note first that *med2*Δ produced the most severe overall recruitment defects seen among all of the mediator mutants that we tested. Second, it is possible that deletion of Med2p indirectly impairs the functions of both Gal11p and Pgd1p, thus disabling the entire tail domain. Finally, Med2p may have a unique function that cannot be carried out by other coactivators.

An alternative explanation for the weak effects of *gal11*Δ and *pgd1*Δ on transcriptional induction, which could also explain the high-level transcription observed in *sin4*Δ cells, is prompted by our finding that these mutations produce partial (*gal11*Δ and *pgd1*Δ) or nearly complete (*sin4*Δ) derepression of *ARG1* transcript levels under noninducing conditions. In addition, they suppress, rather than exacerbate, the transcription defects seen in *med2*Δ cells (Fig. 6B). Others have reported that inactivation of Sin4p, Gal11p, or Pgd1p derepresses transcription from other promoters lacking a UAS, in mutants defective for a coactivator, or under conditions where an activator is nonfunctional (reviewed in reference 30). These findings suggest that these three tail subunits function in repressing basal promoter activity even though they are required for optimal induction by the activator (27), such that deletions of these proteins have offsetting positive and negative effects on transcription. In this view, the loss of recruitment and coactivator functions would outweigh the disruption of repressing activity only in the *sin4*Δ *gal11*Δ and *sin4*Δ *pgd1*Δ double mutants, where we observed a marked reduction in transcription. Perhaps the mediator complexes lacking only Gal11p or Pgd1p function more effectively than WT mediator in promoter clearance, as suggested above for the *sin4*Δ mediator. The repressor activity of Sin4p might also be related to its ability to destabilize reinitiation complexes on immobilized promoters in cell extracts (36). An intriguing possibility is that the tail domain exerts an inhibitory effect on the head or middle domains of mediator through the known physical contacts between these two domains (3, 7) in order to prevent promiscuous transcription in the absence of activators. According to this model, deletion of Sin4p, Gal11p, or Pgd1p would weaken the negative function of the tail domain and bypass the need for Gcn4p to overcome the repressor function of mediator. Presumably, Med2p does not participate in this negative regulatory function and is required only in the recruitment and coactivator functions of mediator. The fact that the *sin4*Δ, *gal11*Δ, and *pgd1*Δ mutations partially suppress the induction defect in *med2*Δ cells implies that one role of Med2p is to overcome the negative function of these other tail domain subunits in the presence of induced levels of activator Gcn4p.

Our finding that *ARG1* mRNA levels are constitutively elevated in the *sin4*Δ mutant (Fig. 6) may seem at odds with the ChIP data showing strong Gcn4p-dependent increases in both TBP binding to the promoter and Pol II association with the ORF in *sin4*Δ cells (Fig. 7A and C). However, this discrepancy can be explained in several ways. It is possible that the high-level *ARG1* transcription seen in uninduced *sin4*Δ cells (Fig. 6B) is still dependent on the basal level of Gcn4p binding to the *ARG1* promoter that occurs in nonstarvation conditions.

Indeed, the uninduced level of Gcn4p makes a strong contribution to *ARG1* transcription in nonstarved WT cells (6). It is also possible that the low-level recruitment of TBP and Pol II afforded by uninduced levels of Gcn4p is sufficient for high-level *ARG1* transcription when the repressing functions of the mediator tail domain are inactivated by deletion of Sin4p.

In summary, our results provide strong evidence that the mediator tail domain is an in vivo target of Gcn4p. Gal11p, Med2p, and Pgd1p are necessary for efficient recruitment of the head and middle domains of mediator and are sufficient for high-level recruitment by Gcn4p as an isolated subcomplex in *sin4*Δ cells. We showed that Med2p is critical for TBP and Pol II recruitment, whereas *sin4*Δ has relatively little effect on PIC formation, even though it disrupts association of the Gal11p/Med2p/Pgd1p triad with the rest of mediator. These last findings raise the intriguing possibility that the tail subcomplex has an important coactivator function in TBP recruitment. It will be interesting to determine whether the Gal11p/Med2p/Pgd1p triad serves directly as an adaptor or facilitates the recruitment of another GTF or coactivator with a role in TBP recruitment.

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