Yeast 14-3-3 Protein Functions as a Comodulator of Transcription by Inhibiting Coactivator Functions*

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Background: Combinatorial control of Adr1-dependent gene expression requires binding of 14-3-3 (Bmh) to Adr1.

Results: Bmh-mediated inhibition of gene expression is activation-domain specific and inhibits the formation of a preinitiation complex.

Conclusion: Bmh-mediated inhibition of the Adr1 activation domain is required for combinatorial control of gene expression.

Significance: Bmh acts as a comodulator of gene expression to enforce combinatorial transcription.

In eukaryotes combinatorial activation of transcription is an important component of gene regulation. In the budding yeast *Saccharomyces cerevisiae*, Adr1-Cat8 and Adr1-Oaf1/Pip2 are pairs of activators that act together to regulate two diverse sets of genes. Transcription activation of both sets is regulated positively by the yeast AMP-activated protein kinase homolog, Snf1, in response to low glucose or the presence of a non-fermentable carbon source and negatively by two redundant 14-3-3 isoforms, Bmh1 and Bmh2. Bmh regulates the function of these pairs at a post-promoter binding step by direct binding to Adr1. However, how Bmh regulates transcription after activator binding remains unknown. In the present study we analyzed Bmh-mediated regulation of two sets of genes activated independently by these pairs of activators. We report that Bmh inhibits mRNA synthesis when the second activator is absent. Using gene fusions we show that Bmh binding to the Adr1 regulatory domain inhibits an Adr1 activation domain but not a heterologous activation domain or artificially recruited Mediator, consistent with Bmh acting at a step in transcription downstream of activator binding. Bmh inhibits the assembly and the function of a preinitiation complex (PIC). Gene expression studies suggest that Bmh regulates Adr1 activity through the coactivators Mediator and Swi/Snf. Mediator recruitment appeared to occur normally, but PIC formation and function were defective, suggesting that Bmh inhibits a step between Mediator recruitment and PIC activation.

Transcription is a key step in gene expression that is regulated by both internal and external cues. The basic mechanisms of this important regulatory process are conserved among eukaryotes from unicellular yeast to multicellular mammals. In addition to the need for the general transcription machinery, specific sets of genes are activated and regulated by a distinct set of promoter-specific transcription factors (TFs).2 Studies have revealed that many genes have binding sites for more than one TF and can respond to environmental or intracellular signals by utilizing different combinations of non-redundant TFs, a process called combinatorial control. The apparent purpose of combinatorial control is to ensure that gene expression can be modulated by integrating multiple signals at the promoter. Combinatorial transcription activation could be achieved at multiple steps including cooperative activator binding and/or cooperative activation by the specific set of activators.

Four prototypic transcription factor families, including non-steroid nuclear receptors (1), MADS box-containing proteins (2), SOX proteins (3), and POU factors (4), represent examples of combinatorial regulation of transcription in mammals. An extensively studied combinatorial transcription regulatory circuit in yeast *Saccharomyces cerevisiae* is comprised of a MADS-box transcription regulator Mcm1 and one of its many co-factors. Mcm1 and its co-regulators play a prominent role in mating type determination (5). In most cases, the Mcm1 homodimer binds DNA cooperatively with the help of a distinct set of DNA-binding proteins to differentially regulate a specific set of genes. Another example of combinatorial regulation in *S. cerevisiae* is represented by the HO promoter. HO expression is activated and regulated by sequential recruitment of transcriptional activators and chromatin remodelers that respond to cell cycle signals, ploidy, and mating factors (6, 7). Swi5 binds first in early G1 phase to the HO promoter and initiates a cascade of events through recruitment of the Swi/Snf, SAGA, and Mediator complexes. In late G1, binding of transcription factor SBF (Swi4/Swi6) to promoter-proximal sequences results in the timely expression of HO. A different type of combinatorial regulation is illustrated by the genes of the methionine biosynthetic pathway. These genes are activated by the TF Met4 that binds DNA with the assistance of three DNA-binding proteins (Met31, Met32, and Cbf1), and one protein (Met28) that facil-

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2 The abbreviations used are: TF, transcription factor; PIC, preinitiation complex; DBD, DNA-binding domain; RD, regulatory domain; AD, activation domain; GBD, Gal4-DNA binding domain; CTD, C-terminal domain; IP, immunoprecipitation; RT-qPCR, reverse transcriptase quantitative real-time PCR; hAd, heterologous activation domain; pol, polymerase; TSS, transcription start site; 4tU, 4-thiouracil; TBP, TATA binding protein.
itates the interaction between Met4 and the DNA-binding proteins to regulate different Met pathway biosynthetic genes (8).

Another example of combinatorial control of gene expression occurs in response to glucose depletion (9), which results in activation of the AMP-activated protein kinase homolog, Snf1 (9). Snf1 targets include transcription factors, coactivator subunits, and proteins involved in post-transcriptional gene regulatory processes (10). Snf1 activates two TFs: Adr1, by promoting dephosphorylation (11); and Cat8, by direct phosphorylation (12, 13). In addition, Snf1 stimulates promoter binding of Adr1 by activating the SAGA-associated histone acetyltransferase Gcn5 (14). Adr1 and Cat8 act together to regulate genes required for the use of non-fermentable carbon sources such as ethanol (ADH2, ACS1, and ALD4) and lactate (JEN1 and CYB2) (15). Genes encoding peroxisomal structural proteins and enzymes of β-oxidation are regulated by Adr1 and the heterodimeric transcription factor Oaf1/Pip2 (16–18). The Snf1 dependence of peroxisomal and β-oxidation genes, however, acts solely through Adr1 (16). In both of these examples the promoters have binding sites for both TFs (15), and they (Adr1 and Cat8 or Adr1 and Oaf1/Pip2) co-occupy the promoters of the genes that they regulate. The absence of either factor causes a 10-fold or greater decrease in mRNA abundance, suggesting that transcription is activated synergistically by the combination of both factors (16, 18–20). Activation of Adr1- and Cat8-dependent genes requires only glucose depletion, whereas Adr1 and Oaf1/Pip2 gene activation requires both glucose depletion and a fatty acid such as oleate (21–24). At the ADH2 promoter, Adr1 promotes Cat8 binding, but other genes that require both factors are able to bind Cat8 in the absence of Adr1 (15). No specific requirements for coactivator recruitment by Adr1 and Cat8 were identified (25), suggesting that synergistic activation of transcription might act at a step after coactivator recruitment.

Adr1 activity, but not its promoter binding, is regulated by the essential yeast 14-3-3 proteins known as Bmh (26, 27). There are two functionally redundant Bmh isoforms, Bmh1 and Bmh2 (28). Either Bmh isoform can bind to an Adr1 regulatory domain (RD) (residues 226–240), first identified by ADR1 alleles that confer the ability to activate ADH2 expression in the presence of the repressing carbon source glucose (29). The ADR1 alleles prevent Bmh binding either by reducing phosphorylation of Ser-230 or by preventing another important ADR1-Bmh interaction (30). A Gal4-DBD (GBD)-Adr1-RD fusion protein is able to bind GAL promoters in the presence of Bmh, but transcription is inhibited (26). Inactivating Bmh, or preventing Bmh binding by mutating the Adr1 regulatory domain, allows abundant GAL mRNA accumulation (26). These data indicate that Bmh inhibits Adr1 activity at a step after GAL promoter binding of Gal4-Adr1 and suggest that inhibition might occur at any one of the steps requiring activation domain (AD) function such as coactivator or PIC recruitment. Evidence that Bmh could inhibit PIC activity was obtained in a yeast mutant lacking wild type histone deacetylase activity (hdacΔ). In such strains Adr1 recruited an inactive PIC that could be activated in repressing high glucose conditions by inactivating Bmh or by preventing its binding to Adr1 (26, 31). The observation that ADR1 alleles stimulate transcription from the inactive PIC indicates that Bmh can act directly at the promoter. Further support for this interpretation was the evidence that Bmh co-occupies Adr1-dependent promoters during repressing conditions in the hdacΔ mutant. However, Bmh is also present when the promoter is fully activated by glucose depletion. The latter result suggests that Bmh could have a role in gene activation as well as in maintaining repression (26).

The function that Bmh fulfills at the Adr1-dependent promoters after glucose depletion appears to be related to combinatorial activation of transcription. Genes that require Adr1 and either Cat8 or Oaf1/Pip2 for high levels of expression can be fully activated by Adr1 alone when either Bmh activity is absent or the Bmh binding site in the Adr1 regulatory domain is disrupted (16, 25, 26). The latter observation demonstrates that the effect acts directly through Adr1 and is not due to interaction of Bmh with other proteins such as the Reg1-Glc7 complex (32). These results suggest that Bmh has a novel function as a coactivator of transcription, enforcing combinatorial control on the expression of genes regulated by Adr1 and a second transcription factor. However the molecular mechanisms of this regulation remain unclear.

Here we report that the mechanism of Bmh-mediated regulation is conserved in two different Adr1-activated systems. Bmh-mediated inhibition of transcription in the absence of a coactivating transcription factor appears to block the formation of a normal PIC. Chromatin immunoprecipitation (ChIP) analyses revealed that Bmh does not inhibit Mediator recruitment. Thus, one function of Bmh may be to inhibit a step in PIC formation that occurs after Mediator recruitment.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Plasmid, and Growth Media**—The *S. cerevisiae* strains used in this study are listed in Table 1, and plasmids are listed in Table 2. *S. cerevisiae* cultures were grown in either yeast extract peptone or synthetic medium containing 5% glucose (repressing medium) and lacking the appropriate amino acid or uracil for plasmid selection. For derepression, mid-log phase cells from repressing medium were harvested by centrifugation, washed with water, resuspended into either yeast extract peptone or synthetic medium having 0.05% glucose, and allowed to grow for the times indicated in Figs. 1 and 2 and for the times indicated in the legends for Figs. 4, 7, 8, 10, and 11. Oleate-inducing medium was made by adding 0.5% Tween 40 and 0.1% oleate to the derepressing medium, and induction was done for 1 h. To maintain selection for plasmids containing *TRP1* and/or *URA3*, the synthetic selective medium contained 0.2% casamino acid rather than the standard dropout solution. All *S. cerevisiae* strains were grown at 30 °C unless stated otherwise. The *BMH2* gene was deleted in both the wild type and mutant (*bmh1-ts*) strains. *BMH1* carries temperature-sensitive mutations in the strain *bmh1-ts* (33). The *bmh1–170(ts)* allele is defective for *ADH2* repression at 30 °C, although it can still grow at this temperature (27). Thus, we used 30 °C for all of our experiments to avoid the possible complication of inducing a heat stress at 37 °C, its restrictive growth temperature.

**mRNA Isolation and Quantitative Real-time PCR (RT-qPCR)**—mRNA was isolated from yeast strains grown in either repressing or derepressing medium using the acid phenol method described in Collart and Oliviero (34). Residual DNA in
the RNA preparation was reduced by using TURBO DNA-free™ kit (Ambion RNA) according to the manufacturer’s recommendations. cDNA was synthesized using an iScript™ cDNA synthesis kit (Bio-Rad) following the manufacturer’s protocol. RT-qPCR was performed for measuring mRNA levels using SsoFast™ EvaGreen® Supermixes (Bio-Rad), diluted cDNA, and specific primer pairs. A standard curve was generated with either ACT1 or Escherichia coli 16S ribosomal (r)DNA primers and used to quantify the mRNA levels. Samples were prepared from biological triplicates and analyzed in duplicate.

Chromatin Immunoprecipitation (ChIP)—ChIP was performed as described in Biddick et al. (25). In brief, repressed cells and cells derepressed for 3–4 h from a 50-ml culture at $A_{600}=1$ were pelleted at 1600 × g at room temperature in a Sorvall RC3B-plus centrifuge and resuspended in 25 ml of 1× PBS supplemented with 1% formaldehyde. After gentle shaking for 15 min at room temperature, 1.5 ml of 2.5 M glycine was added, and the cells were pelleted. The cell pellet was washed once with 10 ml of TBS containing 125 mM glycine and once with 10 ml of TBS only. Protein extract was prepared as described previously (35) after resuspending the cells into ChIP lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate) containing protease (Sigma-Aldrich) and phosphatase inhibitor (Roche Applied Science), the mixture and the clarified extract were then used in subsequent immunoprecipitation (IP) experiments. IP of $\sim$1–2 μg of protein extract (31) was performed for 3 h with constant nutation at 4 °C with specific antibodies and 10 μg/ml sonicated single-stranded salmon sperm DNA. After IP, 50 μl of protein A-coated Mag Sepharose™ Xtra beads (GE Healthcare) was added, and incubation was continued for 1 h at 4 °C. After the beads were separated, 20 μl of the supernatant was removed and mixed with 130 μl of ChIP elution buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 1 mM EDTA). The immunoprecipitated DNA bound to the beads was eluted at 65 °C for 10 min with 150 μl of protein A-coated Mag Sepharose™ Xtra beads (GE Healthcare) was added, and incubation was continued for 1 h at 4 °C. After the beads were separated, 20 μl of the supernatant was removed and mixed with 130 μl of ChIP elution buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 1 mM EDTA). The immunoprecipitated DNA bound to the beads was eluted at 65 °C for 10 min with 150 μl of elution buffer containing 2 μg of sonicated single-stranded salmon sperm DNA. The eluted and input DNA were incubated for 12–16 h at 65°C and then purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer’s protocol. The eluted and input DNA were diluted 10- and 50-fold, respectively, and quantification of specific sequences was performed by RT-qPCR using SsoFast™. Occupancy of a protein is expressed as the fold increase of the IP-to-input ratio of the amount of the specific ampiclon for the

### Table 1

| Strain | Relevant genotype | Reference |
|--------|-------------------|-----------|
| KBY15  | W303–1A MATa cat8Δ-3phMX4 | (26) |
| KBY20  | W303–1A MATa cat8Δ-3phMX4 bmh1Δ::His3::bmh1–170::LEU2 bmh2Δ::KanMX | (26) |
| KBY26  | W303–1A MATa adr1Δ::NatMX bmh1Δ::His3::bmh1–170::LEU2 bmh2Δ::KanMX | (26) |
| KBY30  | W303–1A MATa adr1Δ::NatMX bmh1Δ::His3::bmh1–170::LEU2 bmh2Δ::KanMX | (26) |
| YLL108 | W303–1A MATa bmt2::KanMX | (33) |
| YLL1087| W303–1A MATa bmt1Δ::His3::bmh1–170::LEU2 bmh2Δ::KanMX | (33) |
| PPy1   | W303–1A MATa bmt2::KanMX srb10Δ::NatMex | This study |
| PPy2   | W303–1A MATa bmt2::KanMX srb11Δ::NatMex | This study |
| PPy3   | W303–1A MATa bmt2::KanMX srb10Δ::NatMex | This study |
| PPy4   | W303–1A MATa bmt2::KanMX srb11Δ::NatMex | This study |
| PPy5   | W303–1A MATa bmt2::KanMX srb10Δ::NatMex | This study |
| PPy7   | W303–1A MATa cat8Δ-3phMX4 adr1Δ::NatMX bmh1Δ::His3::bmh1–170::LEU2 bmh2Δ::KanMX | This study |
| PPy9   | W303–1A MATa cat8Δ-3phMX4 adr1Δ::NatMX bmh2::KanMX | This study |
| RBY5   | W303–1A MATa ADR1-5::TRP1 (85::KANMX) | (25) |
| RBY21  | W303–1A MATa ADR1-5::TRP1 (85::KANMX) | (25) |
| RBY30  | W303–1A MATa ADR1-5::TRP1 (85::KANMX) | (25) |
| EAY12  | W303–1A adr1Δ::KanMX gcn5Δ::Htg | (16) |
| EAY15  | W303–1A adr1Δ::KanMX gcn5Δ::Htg | (16) |
| KY50   | W303–1A adr1Δ::KanMX gcn5Δ::Htg | (16) |
| RBY39  | W303–1A MATa adr1Δ::LEU2 cat8Δ::GAL11-5::Htg | (25) |
| RBY42  | W303–1A MATa adr1Δ::LEU2 cat8Δ::GAL11-5::Htg | (25) |

### Table 2

| Plasmid | Marker | Promoter | Gene/Cassette | Reference |
|---------|-------|----------|---------------|-----------|
| Yeast plasmid | | | | |
| pkD16  | TRP1-CEN3 | ADR1   | ADR1 | (59) |
| pkD16H | HIS3-CEN3 | ADR1   | ADR1 | (16) |
| pkD14  | HIS3-CEN3 | ADR1   | ADR1-S230A | (60) |
| pkD14H | HIS3-CEN3 | ADR1   | ADR1-S230A | (16) |
| pPR8   | TRP1-CEN4 | ADH1   | GAL4MN1-ADR1 (215–260)-VP16 (413–490) | This study |
| pPR11  | TRP1-CEN4 | ADH1   | GAL4MN1-ADR1 (215–260)-p53 (1–92) | This study |
| pPR16  | TRP1-CEN4 | ADH1   | GAL4MN1-ADR1 (215–460) | This study |
| pPR27  | TRP1-CEN4 | ADH1   | GAL4MN1-ADR1 (215–260)-GAL11 (797–1080) | This study |
| pKVR1-GAL11 | TRP1-CEN4 | ADH1   | GAL4MN1-ADR1 (215–260)-GAL11 (797–1080) | This study |
| E. coli plasmids | | | | |
| pGEX-3X-BMH1 | | | | |
| pGEX-3X-BMH1 | Ampr | Ptac | BMH1 | (33, 39, 41) |
gene sequence over the IP-to-input ratio corresponding to the amplicon for the telomeric sequence. Primers used for ChIP analysis are listed in Table 3.

RNA Labeling and Calculation of mRNA Synthesis Rate—mRNA synthesis rates were evaluated using the methods and equations described in Miller et al. (36) by labeling the nascent

| Gene | Position | Oligo 5′ to 3′ Sequence |
|------|----------|-------------------------|
| **ADH2** | Promoter | ADH2pQ1 ACATCTACGAGAAGCATGA |
|       |          | ADH2pL AAAAGCTCGACAGGACCTT |
|       | TSS      | KBI17-ADH2F-TSS ACTGCCGTTGAGATTCGATG |
|       | TSS      | KBI18-ADH2R-TSS ACTGCCGTTGAGATTCGATG |
|       | 3′-ORF   | LL5-ADH3QA GTTAGCGCTTGGTCTATT |
|       | 3′-ORF   | LL6-ADH3QB |
| **ACS1** | Promoter | ACS1-C CACCTACGTTGAGATTCGATG |
|       |          | ACS1-B TATTTCCGACAGGACCTT |
|       | TSS      | KBI111-ACS1F-TSS ACTGCCGTTGAGATTCGATG |
|       | TSS      | KBI122-ACS1R-TSS ACTGCCGTTGAGATTCGATG |
|       | 3′-ORF   | LL9-ADH3QA GTTAGCGCTTGGTCTATT |
|       | 3′-ORF   | LL10-ADH3QB |
| **ADY2** | Promoter | KB73-ADY2p-F CCCCAGACTTTCCCTATT |
|       |          | KB74-ADY2p-R ACTGCCGTTGAGATTCGATG |
|       | TSS      | KBI19-ADY2F-TSS ACTGCCGTTGAGATTCGATG |
|       | TSS      | KBI120-ADY2R-TSS ACTGCCGTTGAGATTCGATG |
|       | 3′-ORF   | LL9-ADH3QA GTTAGCGCTTGGTCTATT |
|       | 3′-ORF   | LL10-ADH3QB |
| **FDH** | 3′-ORF   | GL209 CCCAGCTGAAGAGAGATTCGATG |
|        |          | GL210 ATCTCCGACAGGACCTT |
|        | 3′-ORF   | GL29 AAATTTCCGACAGGACCTT |
|        |          | GL30 TCGGCGACTTAAATGCTT |
| **ALD4** | 3′-ORF   | GL29 AAATTTCCGACAGGACCTT |
|        |          | GL30 TCGGCGACTTAAATGCTT |
| **CYB2** | 3′-ORF   | GL281 AATGCGAGAGAGCTT |
|        |          | GL282 AATGCGAGAGAGCTT |
| **JEN1** | 3′-ORF   | LL1-JEN1QA GCCTGGTACCTTTTATCC |
|        |          | LL2-JEN1QB GCTGAGATTCGCTT |
| **ICL1** | 3′-ORF   | LL83 CAGCTGACGATCCACACTTAC |
|        |          | LL84 CAGCTGACGATCCACACTTAC |
| **MLS1** | 3′-ORF   | LL7-MLS1QA TCGGATATCGGGTTGAGTAC |
|        |          | LL8-MLS1QB CAGCTGACGATCCACACTTAC |
| **FBP1** | 3′-ORF   | LL11-FBP1QA ACCCTCTACTGGAACGAGA |
|        |          | LL12-FBP1QB TCTTCTTGTCATGGGTTTA |
| **SIP4** | 3′-ORF   | SIPS_qF TAACTTCTATGCTTATAAAC |
|        |          | SIPS_qR GAGCTGACGATCCACACTTAC |
| **SPS19** | 3′-ORF   | GL271 CAGCTGACGATCCACACTTAC |
|        |          | GL272 CAGCTGACGATCCACACTTAC |
| **CTA1** | Promoter | CTA1-CQF ACCCTCTACTGGAACGAGA |
|        |          | CTA1-CQR GCTGAGATTCGCTT |
|        | 3′-ORF   | GL259 AATGCGAGAGAGCTT |
|        |          | GL260 AATGCGAGAGAGCTT |
| **FOX2** | Promoter | FOX2-CQF ACCCTCTACTGGAACGAGA |
|        |          | FOX2-CQR GCTGAGATTCGCTT |
|        | 3′-ORF   | GL271 CAGCTGACGATCCACACTTAC |
|        |          | GL272 CAGCTGACGATCCACACTTAC |
| **POX1** | Promoter | POX1-CQF ACCCTCTACTGGAACGAGA |
|        |          | POX1-CQR GCTGAGATTCGCTT |
|        | 3′-ORF   | GL189 GCTGAGATTCGCTT |
|        |          | GL190 GCTGAGATTCGCTT |
| **POT1** | Promoter | POT1-CQF ACCCTCTACTGGAACGAGA |
|        |          | POT1-CQR GCTGAGATTCGCTT |
|        | 3′-ORF   | LL27 GCTGAGATTCGCTT |
|        |          | LL28 GCTGAGATTCGCTT |
| **FAA2** | 3′-ORF   | FAA2-QPF GCAACATCTGCTGAGATAGA |
|        |          | FAA2-QPR TGGTATATGCTGAGATAGA |
| **lacZ-1** | ORF      | KBI123.LacZ-1F TGGTATATGCTGAGATAGA |
|         |          | KBI124.LacZ-1R TGGTATATGCTGAGATAGA |
| **lacZ-3** | ORF      | KBI127.LacZ-3F TGGTATATGCTGAGATAGA |
|         |          | KBI128.LacZ-3R TGGTATATGCTGAGATAGA |
| **lacZ-6** | ORF      | KBI133.LacZ-6F AACTTCTTCTATGCTTATAAC |
|         |          | KBI134.LacZ-6R CAGCTGACGATCCACACTTAC |
| **GAL1** | TSS      | PP101 GCCGAGTACGTACCATGAC |
|        |          | PP102 GCCGAGTACGTACCATGAC |
|        | 3′-ORF   | KB9-GAL1.f1 TTCGCCATGACGTACCATGAC |
|        |          | KB10-GAL1.r2 TGGTATATGCTGAGATAGA |
TABLE 3 —continued

| Gene      | Position | Oligo                        | 5′ to 4′ Sequence |
|-----------|----------|------------------------------|-----------------|
| GAL7      | Promoter | KB3-GAL7.f1                  | TTTGATGCTTTCATGGCA |
|           |          | KB4-GAL7.r1                  | GCTCCGCTGAAGTTGTTCA |
|           | TSS      | PP107                        | GCCGCTGGCTGACGTTCA |
|           | 3′-ORF   | KB13-GAL7.f2                 | GTCTTCGGATGTGTTCA |
|           |          | KB14-GAL7.r2                 | GAGCCATGCTGATTTTA |
| GAL10     | Promoter (GAL1–10) | KBI-GAL1.f1 | TGGTCGGACAGTGGCCGCG |
|           |          | KB2-GAL1.r1                  | AGGTCATAGCTCATGCTC |
|           | TSS      | PP103                        | GTGTTAGGCTGCTCCGAT |
|           | 3′-ORF   | KB11-GAL10.f1                | TCCGACGCTTTCGTCAC |
|           |          | KB12-GAL10.r1                | ACCGAGATATATGCTTCC |
| ACT1      | 3′-ORF   | KB5-ACT1.f1                  | CGGCCATTCTCTCCAATC |
|           |          | KB6-ACT1.r1                  | CGAAGATTGACCTTTTCTTC |
| TEL       |          | TEL55                        | GCGTTAACAACACCTAAGCTTCC |
|           |          | TEL56                        | CTCGTTAGATACGCTTCC |

transcript with 4-thiouracil (4tU) (37). Briefly, *S. cerevisiae* cells were grown in synthetic medium supplemented with 5% glucose, 0.2 mM adenine, 0.2% casamino acids, and 0.05 mM uracil to mid-log phase (A600 = 0.8), and then cells were shifted to the derepression media containing 0.2 mM adenine, 0.2% casamino acids, 0.05 mM uracil, and 0.05% glucose and allowed to grow for 3 h. Then cells were incubated with 0.2 mM 4tU (from 2 M stock in dimethyl sulfoxide) for 10–20 min to label the newly synthesized transcripts. After the pulse, cells were harvested by centrifuging at 1600 × g at 4 °C in a Sorvall RC3B-plus centrifuge. Total RNA was extracted as described in Collart and Oliviero (34). Labeled RNA was biotinylated and purified using streptavidin-Mag Sepharose™ beads (GE Healthcare) (38). Prior to the biotinylation reactions, 2 μg of 4-thiouracil labeled *E. coli* total RNA was added to 100 μg of total RNA sample as an internal reference for modification, purification, and cDNA synthesis. The nonbound fraction from the streptavidin beads was collected and used as unlabeled RNA (B). cDNA synthesis was performed with 100 ng of RNA originating from the total, unlabeled, and labeled mRNA using the iScript™ cDNA synthesis kit (Bio-Rad) as described in the manufacturer’s protocol. qRT-PCR was done to quantify the diluted cDNA. To calculate the synthesis rate, we used the equations described in Miller et al. (36) by considering the steady state equilibrium between mRNA synthesis and decay: \( A_g/C_g = 1 - e^{-t(\alpha + \lambda_g)} \), where \( A_g \) is the newly synthesized labeled mRNA amount of gene “g” at time “t” and \( C_g \) is the total amount of mRNA of the same gene at the same time. Here, \( \alpha \) is a factor associated with the growth rate of cells. We considered that it was constant due to the negligible growth during the pulse in derepressing growth conditions. \( \lambda_g \) is the decay rate (molecules/cell/min) of the mRNA corresponding to gene g. The synthesis rate is defined by the equation \( \mu_g = C_g(\alpha + \lambda_g) \).

Analysis of Glucose-induced mRNA Decay—The rate of mRNA decay was measured after adding glucose to a final concentration of 5% to a 4-h derepressed cell culture. The cell culture was collected in 10-mI aliquots in triplicate at 0, 5, 10, 20, and 30 min. Total RNA isolation and mRNA analysis was performed as described above.

**Nascent Transcript Isolation by RNA ChIP**—ChIP was done with rabbit polyclonal anti-Rpb3 antibody (custom-raised against full-length *E. coli*-produced yeast His6-Rpb3 from R&R Research, LLC; a gift from S. Hahn) using a formaldehyde-cross-linked cell extract of 2 h-derepressed samples as described above. During IP, protein extract was incubated with antibody in the presence of RNaseOUT™ ribonuclease inhibitor (RNase, Invitrogen) and 10 μg/ml *E. coli* total RNA. In an RNA-ChIP reaction, RNA was purified from the reverse cross-linked eluate using phenol:chloroform (1:1) extraction followed by chloroform extraction and finally precipitation of nucleic acids with an equal volume of isopropanol in the presence of one-tenth volume of 5 M NaCl and 10 μg of glycogen at room temperature for 10 min. The nucleic acid pellet was collected by centrifuging the suspension for 10 min at 4 °C at 13,000 × g. The pellet was washed with 80% ethanol, dried in a DNA120 SpeedVac® (Thermo Scientific Savant) for 20 min, and dissolved in 20 μl of nuclease-free water. The nucleic acid concentration was measured using a NanoDrop 2000 instrument (Thermo Scientific). 250 ng of the purified nucleic acid sample was freed from DNA using a TURBO DNA-free™ kit (Ambion RNA®, Invitrogen) according to the manufacturer’s protocol. From the DNase-treated sample 100 ng of RNA was used for cDNA synthesis using the iScript™ cDNA synthesis kit (Bio-Rad) as described in the manufacturer’s protocol. qPCR was done to quantify the diluted cDNA.

**GST Pulldown Assays**—Glutathione S-transferase (GST) pulldown assays were done following the protocol described by Parua et al. (27). GST-Bmh1 was expressed from pGEX-3X-BMH1 (39) (a gift from S. Zheng) in *E. coli* BL21(DE3). The fusion protein was immobilized on glutathione-Sepharose 4B beads as described by the manufacturer (GE Healthcare Life Sciences). Pulldown assays were performed using 30–40 μg of glutathione-Sepharose 4B-coupled GST fusion protein and yeast extract containing ~2 mg of total proteins in 1 × PBST (phosphate-buffered saline containing 0.1% Tween 40), 1 × protease inhibitor, and 1 × phosphatase inhibitor (Sigma). Protein extracts from yeast cells were prepared following the procedure described by Parua et al. (27). The suspension was incubated at 4 °C for 1 h with continuous nutation. The beads were then pelleted by centrifugation at 3200 × g for 1 min, washed three times with 1 × PBST, and resuspended in 60 μl of 2 × LDS-NuPAGE sample buffer followed by heating at 95 °C for 5 min. Fractions collected at different steps of the pulldown assays were analyzed by SDS-PAGE followed by Western blot-
ting. Western blot analyses were performed according to the manufacturer’s instructions for the Odyssey infrared imaging system (LiCor Biosciences). Proteins on the PVDF membrane were visualized using diluted (1:500) an anti-Gal4 DNA-binding domain monoclonal antibody (sc-510) and a LiCor/H1800 (1:2500) secondary antibody.

RESULTS

Bmh Regulates Combinatorial Transcription—Peroxisome biogenesis and β-oxidation genes are activated by a heterodimeric activator system, Oaf1/Pip2, with the essential help of Adr1 (16–18, 40). Adr1 has been found to induce the expression of peroxisome biogenesis and β-oxidation genes in response to the fatty acid oleate either by facilitating Oaf1/Pip2 binding to the promoters or by mediating the expression of Pip2 (41, 42). We asked whether Bmh could regulate Adr1-Oaf1/Pip2 combinatorial transcription as we observed for genes activated by Adr1 and Cat8 (26). To test this idea, we analyzed transcripts levels of peroxisome-related genes in response to a 1-h oleate induction in various yeast mutant backgrounds: YLL908 (WT), YLL1087 (bmh1-ts), SRY1 (oaf1Δ), and PPY18 (oaf1Δ bmh1-ts)) in derepressing (0.05% of glucose) and oleate (0.1%) inducing conditions. Oleate induction was done for 1 h. Values were plotted as a percentage of message abundance in wild type cells after normalization them against ACT1 mRNA. B, mRNA analysis was performed in four different S. cerevisiae strains (YLL908 (WT), KBY15 (cat8Δ), KBY20 (cat8Δ bmh1-ts), and YLL1087 (bmh1-ts)) (See also Table 1) grown in low glucose media (0.05%) for 4 h. Values were plotted as a percentage of message abundance in CAT8 BMH1 strains for various Adr1- and Cat8-activated and Cat8 only-activated genes after normalization against ACT1 mRNA. C, observed level of ADH2 and MLS1 (solely Cat8-activated) mRNA after normalization against ACT1 mRNA in four different S. cerevisiae strains (KBY26 (adr1Δ), PPY29 (adr1Δ cat8Δ), PPY27 (adr1Δ cat8Δ bmh1-ts), and KBY30 (adr1Δ bmh1-ts)) in the presence of extrachromosomally expressed Adr1 (pKD16), Adr1c (pKD14-TRP1; Adr1-S230A), or control parental plasmid (pRS314) grown in low glucose medium (0.05%) for 4 h. Error bars represent values obtained from two independent experiment using three biological replicates.

FIGURE 1. Regulation of combinatorial transcription by Bmh. A, expression of various Adr1- and Oaf1/Pip2-activated genes (peroxisome biogenesis and β-oxidation genes) in different S. cerevisiae mutant backgrounds (YLL908 (WT), YLL1087 (bmh1-ts), SRY1 (oaf1Δ), and PPY18 (oaf1Δ bmh1-ts)) in derepressing (0.05% of glucose) and oleate (0.1%) inducing conditions. Oleate induction was done for 1 h. Values were plotted as a percentage of message abundance in wild type cells after normalization them against ACT1 mRNA. B, mRNA analysis was performed in four different S. cerevisiae strains (YLL908 (WT), KBY15 (cat8Δ), KBY20 (cat8Δ bmh1-ts), and YLL1087 (bmh1-ts)) (See also Table 1) grown in low glucose media (0.05%) for 4 h. Values were plotted as a percentage of message abundance in CAT8 BMH1 strains for various Adr1- and Cat8-activated and Cat8 only-activated genes after normalization against ACT1 mRNA. C, observed level of ADH2 and MLS1 (solely Cat8-activated) mRNA after normalization against ACT1 mRNA in four different S. cerevisiae strains (KBY26 (adr1Δ), PPY29 (adr1Δ cat8Δ), PPY27 (adr1Δ cat8Δ bmh1-ts), and KBY30 (adr1Δ bmh1-ts)) in the presence of extrachromosomally expressed Adr1 (pKD16), Adr1c (pKD14-TRP1; Adr1-S230A), or control parental plasmid (pRS314) grown in low glucose medium (0.05%) for 4 h. Error bars represent values obtained from two independent experiment using three biological replicates.
inhibition in the presence of Oaf1 causes enhanced activation of combinatorially activated genes due to the combined functions of the two activators, Adr1 and Oaf1.

We extended our studies to evaluate the role of Bmh to regulate the expression of seven Adr1- and Cat8-activated genes (ADH2, FDH, ADY2, ACS1, FOX2, ALD4, and JEN1) in four different strains (WT (YLL908), cat8Δ (KBY15), cat8Δ bmh1-ts (KBY20), and bmh1-ts (YLL1087)) after 4 h of derepression. Fig. 1B illustrates the influence of Bmh on the expression of Adr1- and Cat8-activated genes. Transcript abundance was reduced to ∼1–25%, depending on the promoter activity in a strain lacking CAT8, and its abundance was restored to higher than WT level in a cat8Δ bmh1-ts double mutant strain. These results suggest that Cat8 is required for suppressing the inhibition that occurs when Bmh is bound to Adr1. In bmh1-ts cells, where all the activators were functionally active, gene expression was increased further (~2-fold) compared with that observed in the WT and the double mutant strain. The regulation of combinatorial transcription by Bmh happens predominantly through direct binding to Adr1, as confirmed by the efficient binatorial transcription by Bmh happens predominantly through direct binding to Adr1, as confirmed by the efficient

To determine whether the relief from Cat8 dependence occurs throughout the time course of glucose depletion, we measured the derepression kinetics of three genes (ADH2, ACS1, and ICL1) (Fig. 2, A–C). The expression of both ADH2 and ACS1 increased with time after glucose depletion, and comparable amounts of mRNA were observed after 4 h in the wild type and double mutant strains (Fig. 2, A and B). In the cat8Δ strain the transcript abundance increased very slowly with time and was reduced 90–98% after 4 h compared with that observed in the WT and cat8Δ bmh1-ts double mutant strains. The relative mRNA abundance, WT/mutant, was comparable between 1 and 4 h, suggesting that Bmh has a continuous inhibitory effect in the absence of Cat8. The expression of ICL1, a solely Cat8-activated gene, did not increase after glucose depletion in the cat8Δ strain and increased gradually with time in the WT (Fig. 2C). The restoration of Adr1-dependent gene expression in the cat8Δ bmh1-ts double mutant was not due to the activation of Sip4, a paralog of Cat8 that is transcriptionally activated by Cat8 in a Snf1-dependent way (43), because we observed Sip4 expression only in the wild type strain (Fig. 2D). Moreover, Sip4 mRNA was detected only after 2 h of glucose depletion, whereas an increase in ADH2 and ACS1 mRNA abundance was observed after 15 min (Fig. 2, A and B). In conclusion Bmh-mediated regulation of combinatorial transcription is conserved between two sets of Adr1-activated genes and is achieved through direct binding to Adr1.

**mRNA Synthesis Rates and pol II-Associated Nascent Transcripts Are Reduced by Bmh in the Absence of CAT8**—To determine the level of gene expression that is inhibited by Bmh in the absence of CAT8 we measured the transcription rate and amount of elongating RNA pol II-associated nascent transcripts of Adr1- and Cat8-activated genes. To measure the

![Figure 2](image-url)
mRNA synthesis rate, we performed 4tU labeling of the nascent transcripts for 10 and 20 min after growing cells in low glucose (0.05%) medium for 2 h. The 4tU-containing RNA molecules can be modified by biotinylation and purified by binding to a streptavidin column. Thus, one can measure an apparent synthesis rate at the end of the pulse. The outline of the experiment is shown in Fig. 3A. We processed total RNA, unlabeled RNA (the nonbound fraction from the streptavidin column), and purified 4tU-containing RNA for cDNA synthesis and performed RT-qPCR. We measured mRNA synthesis rates for three genes, ADH2, ACS1, and ADY2, using the method described by Miller et al. (36) that evaluates steady state mRNA synthesis and decay. The equations used to evaluate the synthesis rate are indicated under “Experimental Procedures” above. As shown in Table 4, the mRNA synthesis rates in cat8Δ cells were ~10–50-fold lower than those observed in WT and cat8Δ bmh1-ts cells, respectively, depending on the promoter. The total level of transcripts for the three genes varied in a similar manner. Therefore, the decreased rate of mRNA synthesis in the cat8Δ versus WT and cat8Δ bmh1-ts strains agrees with
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TABLE 4
mRNA synthesis rates

| Gene   | WT/cat8Δ | cat8Δ bmh1-ts/cat8Δ |
|--------|-----------|---------------------|
| ADH2   | 14        | 57                  |
| ACS1   | 12        | 18                  |
| ADY2   | 12        | 11                  |

reduced transcript abundance in the same strains and suggests that a transcriptional defect in cells devoid of the second activator, Cat8, may be solely responsible for the low abundance of transcripts. In an independent experiment we measured the amount of nascent transcripts associated with the elongating RNA pol II and obtained additional results suggesting that the combinatorial regulation by Bmh is due to a transcriptional defect in the absence of Cat8. We followed the normal ChIP for total pol II using anti-Rpb3 antibody. However, we isolated and purified only the nascent transcripts associated with elongating pol II by treating the sample with DNase I. Following cDNA synthesis and RT-qPCR (Fig. 3B), we assayed the cDNAs corresponding to two endogenous genes (ADH2 and ACS1) and a genome-integrated ADH2p-lacZ reporter cassette (Fig. 3C). As a negative control, RNA was similarly labeled, purified, and characterized from cells grown under glucose-replete, repressed growth conditions, where these genes were not actively transcribed. As shown in Fig. 3D the amounts of nascent transcripts associated with pol II were very low (reduced ~10–50-fold) at the 3’-end of ADH2 and ACS1 and also along the lacZ ORF in cat8Δ cells compared with that observed in the double mutant and WT cells. As expected, nascent transcripts were not detected in mRNA purified from repressed cells. In conclusion, both mRNA synthesis rates and the amount of elongating pol II-associated nascent transcripts suggest that Bmh is responsible for a transcriptional defect in the absence of the second activator in a combinatorially regulated system.

**Bmh Does Not Influence mRNA Stability**—To confirm that gene expression is regulated by Bmh primarily and perhaps exclusively at a transcriptional as opposed to a post-transcriptional step by Bmh, we analyzed mRNA stability in the presence and absence of Bmh. The yeast AMP-activated protein kinase Snf1 plays an important role in stabilizing Adr1- and Cat8-activated transcripts (44). Bmh regulates Snf1 activity via Reg1 (the regulatory subunit of the type 1 protein phosphatase Glc7), and it has been hypothesized that upon binding to Reg1, Bmh increases the phosphatase activity of Glc7, which in turn inactivates Snf1 by dephosphorylating pThr-210 in the activation loop (32). Thus, we asked whether Bmh functions via Snf1 to regulate the stability of glucose-repressed mRNA. To examine this possibility, we measured mRNA abundance as a function of time after glucose was restored to a derepressed culture of BMH1 WT and bmh1-ts mutant cells (Fig. 4A). RNA pol II is rapidly lost from the promoter and gene body when glucose is added to a derepressed culture, effects that are due at least in part to the inactivation of Snf1 (44). Thus, mRNA stability can be assessed without the complication of continued RNA synthesis. Using this protocol we showed that both ADH2 and ACS1 transcripts displayed comparable stability in WT and mutant strains, demonstrating that Bmh does not regulate mRNA stability (Fig. 4B). Therefore, our assays of mRNA abundance in the presence and absence of Bmh are indicative of differences in mRNA synthesis and not mRNA decay.

**Bmh-mediated Regulation Is Activation Domain-dependent**—Conservation of Bmh-mediated regulation in two independent systems, Adr1-Cat8 and Adr1-Oaf1, suggests that Bmh functions through Adr1, the Bmh binding partner in these systems. To determine whether the RD (residues 215–260) of Adr1 is sufficient to confer Bmh-mediated regulation on gene expression, we replaced the Adr1 activation domain (Adr1AD; residues 260–460) with three heterologous activation domains (hADs), the C-terminal region (residues 797–1081) of yeast Gal11, a subunit of Mediator (Med15), which acts as an artificial activator (45), human p53 (residues 1–92) (46, 47), and HSV-encoded transcription activator VP16 (residues 411–490) (48, 49). The chimeric activators were expressed as GBD fusion proteins in both the wild type and bmh1-ts mutant strains in the presence of the Bmh RD binding region (residues 215–260). The fusion constructs are diagrammed schematically in Fig. 5A. Their activity was evaluated by measuring the expression of Gal4-dependent genes (GAL1, GAL7, and GAL10). Fig. 5B presents GAL expression profiles in BMH1 and bmh1-ts mutant strains expressing the GBD fusion activators. Chimeric activators with VP16, p53, and Gal11 showed comparable levels of activity in BMH1 wild type and bmh1-ts mutant strains, suggesting that Bmh has a small or negligible inhibitory role in these activation domains. In contrast Adr1AD showed ~20–40 fold inhibition of activity in the wild type strain compared with that in bmh1-ts strain (Fig. 5B), suggesting that Bmh inhi-
bition is conferred through the Adr1 activation domain. The AD specificity is not due to the level of expression and strength of the AD, because we observed comparable amounts of all of the fusion hADs (Fig. 5A, left panel); and in the absence of Bmh activity, Adr1AD was as active as the non-inhibited hADs. To confirm that Bmh could bind the RD in the context of chimeric activators, we assessed Bmh binding to all of the GBD fusion activators by GST pulldown assays using glutathione-Sepharose 4B-immobilized GST-Bmh1 and yeast extracts expressing a fusion peptide. As shown in Fig. 5C, Bmh retained the ability to interact with all of the GBD fusion activators, suggesting that despite the target binding, Bmh is unable to inhibit the activity of two heterologous activation domains or an artificially recruited Mediator.

To determine whether the lack of inhibition of artificially recruited Mediator was influenced by the GBD, we tested the

FIGURE 5. Bmh-mediated regulation is specific for the Adr1 activation domain. A, schematic diagram of GBD fusion hADs (the C-terminal region (residues 797–1081) of yeast Gal11, human p53 (residues 1–92), HSV-encoded transcription activator VP16 (residues 411–490), and Adr1 (residues 260–460). Left panel, represents the level of abundance of the corresponding fusion activator. Results were obtained by Western blotting of the cell extracts with anti-GBD monoclonal antibody (sc-510). B, abundance of GAL mRNAs (GAL1, GAL7, and GAL10) in Bmh1 WT and bmh1-ts mutant strains for four different heterologous AD. Values were plotted after normalization against ACT1 mRNA. C, GST pulldown of four different GBD fusion activators having a Bmh binding region (RD) with glutathione-Sepharose 4B-immobilized GST-Bmh1 or GST. Cell extract (CE) and pellet (P) fractions were electrophoresed on Any kD™ Mini PROTEAN TGX gels (Bio-Rad) followed by visualization of proteins with Western blotting using anti-GBD antibody (sc-510). D–F, ADH2 (D), ACS1 (E), and ICL1 (F) mRNA abundance in four different S. cerevisiae strains (KBY26 (adr1Δ), PYP29 (adr1Δ cat8Δ), PYP27 (adr1Δ cat8Δ bmh1-ts), and KBY30 (adr1Δ bmh1-ts)) harboring a plasmid expressing either the C terminus of Gal11 (residues 797–1081) or an analogous plasmid lacking the RD grown in low glucose (0.05%) medium for 4 h. Values were plotted after normalization against ACT1 mRNA. Error bars represent values obtained from two independent experiment using three biological replicates.
ability of Bmh to inhibit Adr1DBD-Gal11 chimeric activator at two Adr1- and Cat8-dependent promoters, ADH2 and ACS1. The effect of Bmh was measured in the presence and absence of the RD in four different strains, adr1Δ (KY26), adr1Δ cat8A (PPY29), adr1Δ cat8A bmh1-ts (PPY27), and adr1Δ bmh1-ts (KY30). As shown in Fig. 5, D and E, there was a comparable level of gene expression independent of the genetic background for each of the fusion proteins (Adr1DBD-Gal11 and Adr1DBD-RD-Gal11). The amount of mRNA for ADH2 and ACS1 was reduced about 2-fold when the fusion protein contained RD, but importantly, for both ADH2 and ACS1 mRNA abundance due to activation by Adr1DBD-Gal11 with or without RD was independent of Bmh binding. The sole Cat8-activated gene ICL1 was expressed only when Cat8 was present (Fig. 5F). These results suggest that Bmh does not regulate combinatorial transcription when a heterologous AD or an artificially recruited Mediator replaces Adr1AD. Therefore the Bmh-mediated regulation of combinatorial transcription is specific for the Adr1AD, further suggesting that regulation is achieved after activator binding.

**Bmh Regulates PIC Formation**—To test the ability of the various ADs to recruit RNA pol II, we performed a ChIP using RNA pol II-specific antibody that recognizes the Rpb3 subunit of pol II. We measured RNA pol II recruitment at Gal4-dependent promoters in the context of various GBS fusion activators having the Adr1 RD (Fig. 5A). Promoter recruitment of RNA pol II when GBD-RD-Adr1AD was the activator was reduced 30–55-fold compared with GBD-RD with hADs from p53 and VP16 (Fig. 6A). The latter two hADs showed no inhibition of RNA pol II recruitment by Bmh. The ChIP results using anti-Rpb3 antibody indicated that Bmh has an important role in RNA pol II recruitment and that its recruitment is influenced by the nature of the AD.

Further support for a role of Bmh in PIC recruitment was obtained using GBD-Adr1AD with or without RD in BMH WT and bmh1-ts strains. In the presence of the Bmh binding region (RD), RNA pol II recruitment was reduced ~10–12-fold compared with that observed for the fusion activator without the RD (Fig. 6B). Furthermore there was a comparable level of RNA pol II occupancy at the GAL promoters (GAL7 and GAL10) in a bmh1-ts strain both in the presence and absence of the RD (Fig. 6B). Consistent with our previous observations (26) the fusion activators were recruited with comparable efficiency at Gal4-dependent promoters in functional and nonfunctional Bmh backgrounds and in the presence and absence of the Bmh binding region (RD) (Fig. 6, C and D). These results suggest that Bmh does not have an effect on promoter binding by Adr1 but can regulate PIC formation by regulating the recruitment of pol II.

To investigate the involvement of Cat8 in pol II recruitment in the presence and absence of Bmh function, we measured RNA pol II recruitment by using anti-Rpb3 antibody at Adr1-dependent promoters in response to low glucose in wild type cells or in cells lacking Cat8 in combination with WT BMH1 and bmh1-ts. As shown in Fig. 7A, at the ADH2, ACS1, and ADY2 transcription start site (TSS), RNA pol II recruitment was reduced significantly (~6–10-fold) in cat8Δ cells compared with that observed in the WT and double mutant (cat8Δ bmh1-ts) strain, suggesting that in the absence of Cat8, Bmh inhibits RNA pol II recruitment and subsequent transcription initiation. The presence of Cat8 appears to suppress this inhibitory step. We next demonstrated that Oaf1 plays an analogous role at promoters activated by Adr1 and Oaf1/Pip2. As shown in Fig. 7B, at four different Adr1- and Oaf1-activated promoters, POX1p, POT1p, CTA1p, and FOX2p, RNA pol II recruitment was inhibited in the absence of Oaf1 after 2 h of olate
induction. Thus, in both of these co-regulated systems an inhibitory function of Bmh was suppressed by the non-Bmh-binding TF, Cat8, or Oaf1/Pip2. RNA pol II recruitment was further analyzed using another pol II-specific antibody (ab5131, Abcam) that recognizes phosphorylated Ser-5 residues of the heptad repeat (YSPTSPS) of the C-terminal domain (CTD) of

FIGURE 7. Bmh inhibits PIC formation. A, ChIP results showing the amount of RNA pol II detected at the TSS of three Adr1- and Cat8-activated genes (ADH2, ACS1, and ADY2) in WT, cat8Δ, and cat8Δ bmh1-ts cells grown in low glucose (0.05%) medium using anti-Rpb3 antibody. B, ChIP results showing the amount of recruited RNA pol II at the TSS of four Adr1- and Oaf1-activated genes in WT, oaf1Δ, and oaf1Δ bmh1-ts strains grown in low glucose (0.05%) medium under oleate-inducing conditions for 2 h. C and D, abundance of pSer-5-CTD-containing RNA pol II at the TSS of two GAL genes (GAL7 and GAL10) in BMH1 WT and Adr1-dependent promoters. C, ChIP results showing the amount of RNA pol II-CTD pSer-5-recruited pol II at the TSS of two GAL genes (GAL7 and GAL10) in BMH1 WT strain harboring plasmids expressing the p53 activation domain (p53AD, residues 1–92), VP16AD (residues 411–490), or Adr1AD (residues 255–460) as GBD and Bmh binding region (RD) fusion proteins using rabbit polyclonal anti-pSer-5 antibody (ab5131, Abcam). D, ChIP using rabbit polyclonal anti-pSer-5 antibody to detect the pSer-5 level of recruited pol II at the TSS of ADH2, ACS1, and ADY2 in three different S. cerevisiae strains (YLL908 (WT), KBY15 (cat8Δ), and KBY20 (cat8Δ bmh1-ts) grown in low glucose (0.05%) medium. E, ChIP results showing the amount of TFIIF recruitment at the TSS of three Adr1- and Cat8-activated genes (ADH2, ACS1, and ADY2) in WT, cat8Δ, and cat8Δ bmh1-ts cells grown in low glucose (0.05%) medium using rabbit polyclonal anti-Tfg2 antibody (generated against recombinant yeast Tfg2; a gift from S. Hahn). F, ChIP results showing amount of TFIIH recruitment at the TSS of three Adr1- and Cat8-activated genes (ADH2, ACS1, and ADY2) in WT, cat8Δ, and cat8Δ bmh1-ts cells grown in low glucose (0.05%) medium using rabbit polyclonal anti-Adr1 antibody (custom raised against E. coli-produced recombinant yeast Adr1 from R&R Research, LLC; a gift from S. Hahn). G, ChIP results showing the amount of TBP detected at the TSS of two Adr1- and Cat8-activated genes (ADH2 and ACS1) in WT, cat8Δ, and cat8Δ bmh1-ts cells grown in low glucose (0.05%) medium using anti-TBP antibody. H, ChIP results showing the amount of TFIIB detected at the TSS of three Adr1- and Cat8-activated genes (ADH2, ACS1, and ADY2) in WT, cat8Δ, and cat8Δ bmh1-ts cells grown in low glucose (0.05%) medium using anti-TFIIB antibody. WT-R, represents value obtained in WT cells grown under repressing condition (5.0% glucose). Values were plotted after normalization against the telomere region (TEL) amplified using a telomere-specific primer pair (Table 3). Error bars in A–F represent values obtained from two independent experiment using three biological replicates. For G and H, the error bars represent values from three biological replicas of one experiment.
the large subunit (Rpb1) of pol II. Consistent with RNA pol II recruitment, we also observed significantly reduced levels of Ser-5 phosphorylation at the TSSs of GAL genes (GAL7 and GAL10) when GBD-RD-Adr1AD was the activator compared with GBD-RD with hADs from p53 and VP16 (Fig. 7C). We also observed significantly reduced levels of pol II CTD pSer-5 at three Adr1- and Cat8-dependent promoters, ADH2p, ACS1p, and ADY2p, in a cat8Δ strain compared with WT and cat8Δ bmh1-ts cells (Fig. 7D), further suggesting the inhibition of pol II recruitment by Bmh when Cat8 is absent. We tested the possibility that even though RNA pol II appeared to be absent, other general transcription factors might be present at these promoters. As expected, the general TFs TFIIF and TFIHF were less abundant at these promoters in cat8Δ cells compared with those observed in WT and cat8Δ bmh1-ts strains (Fig. 7, E and F). TBP and TFIIF recruitment were also diminished in the cat8Δ cells compared with WT and cat8Δ bmh1-ts strains (Fig. 7, G and H), suggesting that PIC formation was inhibited at an early step. In conclusion, Bmh regulates combinatorial transcription by direct binding to Adr1 by inhibiting PIC formation, an inhibitory function that is suppressed by the second activator, Cat8 or Oaf1.

An undetectable level of recruited RNA pol II and other PIC components in the absence of the second activator (Cat8 or Oaf1) in a combinatorial transcription system might be due to the complete inhibition of pol II recruitment or to the rapid loss of a transiently formed but unstable PIC. To test this possibility, we performed a glucose shutoff experiment as described in Fig. 4A. We grew various mutant yeast cells, (YLL908 (WT), KBY15 (cat8Δ), and KBY20 (cat8Δ bmh1-ts)), to mid-log phase (5.0% glucose), shifted to low glucose (0.05% glucose), and allowed the cells to derepress for 4 h. Glucose was restored (5.0%) to the cultures, and cells were collected at 0, 5, and 10 min and processed for ChIP using anti-Rpb3 antibody. As shown in Fig. 8, there was a rapid loss of recruited pol II from three Adr1- and Cat8-activated promoters (ADH2p, ACS1p, and ADY2p) both in WT and cat8Δ bmh1-ts cells. However, in the absence of Cat8 the level of pol II recruitment was low and comparable to the value observed at the 10-min time point, suggesting that there was low recruitment of pol II in the absence of Cat8. Loss of pol II at the ACS1 and ADY2 promoters appeared to be more rapid in the absence of Bmh activity, suggesting that Bmh might help stabilize the PIC, a possibility that merits further investigation.

The results indicating inhibition of pol II recruitment by Bmh appear to be inconsistent with our previous studies, which support a post-PIC formation role for Bmh (26). In these studies RNA pol II was observed at inactive GAL promoters when GBD-RD-Adr1AD was present in a BMH WT strain (26). The former ChIP analyses were performed with the 8WG16 antibody, which recognizes both non-phosphorylated and Ser-5-phosphorylated pol II CTD. Furthermore, in a histone deacetylase (hda1Δ rpm3Δ, referred to as hdaΔ) mutant, an apparently inactive PIC is recruited by Adr1 under repressing conditions in a BMH WT strain (31). In a bmh1-ts strain, or when Adr1 is the activator, the PIC was active, suggesting that Bmh inhibits PIC activity and not its formation (26). We repeated the ChIP experiment for Gal4-DBD fusion hADs (diagrammed in Fig. 5A) using 8WG16 antibody (ab817, Abcam®). As shown in Fig. 9A, there was a comparable amount of promoter-recruited RNA pol II for all three fusion activators in BMH WT cells, despite very low GAL mRNA abundance in the case of GBD-RD-Adr1AD (Fig. 5B). In an independent experiment using three fusion proteins (GBD-RD-Adr1AD, GBD-Adr1AD, and GBD-RD), a 2-fold lower level of RNA pol II recruitment at two GAL promoters was observed when the fusion activator had the Bmh-binding domain (RD) compared with that observed for the fusion activator without the RD (Fig. 9B). As expected in the absence of an activation region (GBD-RD), there was a very low level of RNA pol II recruitment at the promoters, suggesting that pol II recruitment was achieved through the activator.
Both of these results are consistent with our previously published results and agree with a post-PIC formation role of Bmh in the regulation of Adr1 activity (26). However, our new results using anti-Rpb3, anti-CTD-pSer-5, anti-Tfd2, anti-Ssl2, anti-TBP, and anti-TFIIB antisera in two independent systems, BGD fusion proteins and Adr1-dependent promoters, suggest that Bmh functions at the post-activator binding step to inhibit transcription by inhibiting PIC assembly. A possible explanation of this apparent conundrum is presented under “Discussion” below.

PIC Formation Is Not Regulated through Chromatin Modification—To understand the molecular events through which Bmh inhibits PIC formation, we measured total H3 and H4 acetylation at lysine residues (H3KAc and H4KAc), as the marks have been associated with PIC formation and transcriptional activation (50). Total H3 and H4 acetylation was analyzed by ChIP in two independent systems, at endogenous Adr1- and Cat8-activated promoters and at Gal4-dependent promoters by expressing GBD fusion constructs described above in Fig. 5A. As shown in Fig 10A, the amount of total H3KAc was comparable in wild type, cat8Δ, and cat8Δ bmh1-ts cells at the TSS of two Adr1- and Cat8-activated genes (ADH2 and ACS1), suggesting that Bmh does not function through H3K acetylation to regulate the PIC formation at these promoters. Not only the H3KAc but also the H4KAc was unaffected at three Adr1- and Cat8-activated promoters (ADH2, ACS1, and ADY2), either upon CAT8 deletion or making BMH nonfunctional in the cat8Δ background, further suggesting that Bmh does not act via common acetyltransferases to regulate the PIC formation (Fig 10B). Consistent with this conclusion, we also observed comparable levels of histone acetylation at three Gal4-dependent promoters (GAL1p, GAL7p, and GAL10p) for all three fusion activators (p53, VP16, and Adr1), despite having a significantly lower level of GAL gene expression when Adr1 was the activator (Fig 10, C and D). The similarity of the H3 and H4 acetylation profiles agrees with the modest effect on Gcn5 (N-acetyltransferase) and NuA4 (H4 acetyltransferase) occupancy upon deletion of CAT8 (25). The importance of histone acetylation in PIC formation was also studied using the BGD fusion Adr1AD in the presence and absence of the RD in BMH WT and bmh1-ts. As shown in Fig 10E, there was a comparable level of H3KAc at three Gal4-dependent promoters (GAL1p, GAL7p, and GAL10p), both in the presence and the absence of the Bmh-binding domain (RD), suggesting that although Bmh binding regulates the activity of the fusion activator through the formation of a PIC, H3KAc does not play an important role in that regulation. These results are consistent with histone acetylation of ADIR1-dependent promoters having as its primary function a role in promoter binding of the activators, and not a post-activator function, as appears to be the case for Bmh.

Another important mark of gene activity is histone methylation. Histone H3-K4 trimethylation (H3K4me3) downstream of the start site of transcription is strongly correlated with gene transcription (51). ChIP analysis of ADH2, ACS1, and ADY2 promoters showed that H3K4me3 was reduced to the low level observed in transcriptionally inactive repressed cells (WT-R) in the cat8Δ mutant compared with WT and cat8Δ bmh1-ts cells in cells derepressed for 4 h (Fig 10F). H3K4me3 was also reduced 5–10-fold when the Bmh-sensitive GBD-Adr1AD fusion protein was the activator compared with the level observed when the Bmh-insensitive GBD-VP16 and GBD-p53 fusion activators were present (Fig 10G). These results suggest that recruitment of the Set1 histone methyltransferase is defective in the presence of Bmh when Cat8 is absent. Set1 is recruited by the Ser-5-modified CTD of elongating RNA pol II (51). Thus the results are entirely consistent with a defect in RNA pol II recruitment in the presence of Bmh when Cat8 is absent.

Bmh Impairs Coactivator Function in the Absence of Cat8—To understand whether Bmh influences PIC formation through coactivator function, we analyzed GAL gene expression by expressing heterologous fusion activators having RD (schematically diagrammed in Fig. 5A) in BMH WT strains in three coactivator mutant yeast (gcn5Δ (EAY12), gal11Δ (TYY540), and snf2Δ (EAY15)). As shown in Fig. 11A, the activity of Adr1AD was reduced ~12–20-fold in snf2Δ and gal11Δ strains, whereas the activity of two hADs (VP16 and p53) was reduced only ~2–5-fold in the same strains, suggesting that Bmh might function through the Mediator complex and the Swi/Snf remodeler, either by regulating their recruitment or by modulating there activity. The Adr1AD was reduced in activity about 2-fold in the absence of GCN5, as were the hADs, suggesting that its histone acetyltransferase activity is less important, consistent with the data in Fig 10 showing that histone H3 acetylation at GAL promoters was unaffected by the nature of the AD.

As we had observed that the function of both Mediator and the Swi/Snf complex is required for complete derepression of ADH2 (52), we tested whether Bmh inhibits Mediator recruitment and Cat8 suppresses that inhibition. We analyzed Mediator recruitment at three Adr1- and Cat8-activated promoters, ADH2p, ACS1p, and ADY2p, by ChIP in three different strains, WT, cat8Δ, and cat8Δ bmh1-ts, grown under derepressing conditions. AS a control Mediator recruitment was also assessed in glucose-replete, repressed conditions. As shown in Fig 11B, Mediator recruitment, as assessed by ChIP for the Srb4 (Med17) head subunit, was unaffected by the absence of either Bmh or Cat8. ChIP for two other Mediator subunits, Gal11, a tail component, and Med4, a middle subunit, behaved similarly (data not shown). The results observed in the cat8Δ strain are consistent with previous observations (25). In cat8Δ cells we detected Mediator recruitment, and the amount was comparable with that observed in the WT and the double mutant cells. Furthermore, the binding was significantly higher under derepressing conditions compared with the low level in wild type repressing conditions (Fig. 11B, WT-R). This ChIP result suggests that Bmh does not regulate the Mediator recruitment step, although the GAL gene expression studies using hADs and Adr1AD suggest that Mediator plays an important role in Bmh-mediated regulation.

Mediator has been suggested to exist in two forms, one containing and the other lacking the regulatory module containing a cyclin and a cyclin-dependent kinase, and apparent conversion between these forms may be affected by activator binding (53, 54). This suggests the possibility that Bmh inhibits the switch from a repressive to an activating form of Mediator and that this inhibitory function might be overcome by Cat8. To
test this possibility, we assayed repressed \textit{ADH2} expression in strains lacking subunits of the kinase module, namely, \textit{med12}/\textit{H9004}/\textit{srb8}/\textit{H9004}, \textit{med13}/\textit{H9004}/\textit{srb10}/\textit{H9004}/\textit{cdk8}/\textit{H9004}, and \textit{srb11}/\textit{H9004}/\textit{cycC}/\textit{H9004}. There was no relief from repression in these strains, suggesting that Bmh may not function through the kinase module of Mediator to inhibit \textit{ADH2} expression (Fig 11C). We then attempted to make the same mutations in a strain lacking Bmh activity. We were only able to create a \textit{med12}/\textit{H9004}/\textit{bmh1-ts} strain (unpublished data),
suggesting that there may be an important genetic interaction between Bmh and the cyclin/cyclin-dependent kinase, module of Mediator. In the \textit{med12} \textit{bmh1-ts} strain there was a synergistic increase in \textit{ADH2} expression, suggesting that the kinase module may have a role in repressing \textit{ADH2} expression that is revealed when Bmh is absent.

We measured Swi/Snf occupancy by performing ChIP for Snf2-Myc, a Swi/Snf subunit in RBY5 (WT) and RBY21 (\textit{cat8Δ}), and in RBY30 (\textit{adr1Δ cat8Δ}) expressing the dominant, non-Bmh-binding \textit{ADR1}^{c} allele (Table 2). As shown in Fig 11D, there was an \textasciitilde 40 – 60\% decrease in Swi/Snf occupancy at three Adr1- and Cat8-activated promoters (\textit{ADH2p},

In A, abundance of GAL mRNAs (GAL7 and GAL10) in WT and various coactivator (\textit{gcn5Δ} (EAY12), \textit{gal11Δ} (TYY540), and \textit{snf2Δ} (EAY15)) mutant strains harboring vector expressing one of three different heterologous GBD-RD-AD fusion proteins described in Fig. 5A. Values were plotted after normalization against ACT1 mRNA. B, ChIP to detect Mediator recruitment at the promoter of three Adr1- and Cat8-activated genes (\textit{ADH2}, \textit{ACS1}, and \textit{ADY2}) in WT (YLL908), \textit{cat8Δ} (KBY15), and \textit{cat8Δ bmh1-ts} (KBY20) cells grown in low glucose (0.05\%) medium using rabbit polyclonal anti-Srb4 antibody (generated against recombinant yeast Srb4, a gift from S. Hahn). WT-R, represents values obtained in WT cells grown in repressing medium (5.0\% glucose). C, observed level of \textit{ADH2} message in various Mediator subunit mutant backgrounds (\textit{PPY2} (\textit{srb8Δ}), \textit{PPY3} (\textit{srβ10Δ}), \textit{PPY4} (\textit{srβ11Δ}), \textit{PPY5} (\textit{bmh1-ts srβ8Δ}), \textit{YLL908} (WT), and \textit{YLL1087} (\textit{bmh1-ts})) under repressing conditions (5.0\% glucose). D, occupancy of Swi/Snf complex at Adr1- and Cat8-activated promoters (\textit{ADH2p}, \textit{ACS1p}, and \textit{ADY2p}) was measured by ChIP using anti-cMyc antibody (9E10X) against endogenously expressed \textit{SNF2-MYC13} from three different \textit{S. cerevisiae} strains (RBY5 (WT), RBY21 (\textit{cat8Δ}), and RBY30 (\textit{adr1Δcat8Δ}), harboring vector pKD14H (\textit{HIS3}) expressing \textit{ADR1}^{c} \textit{S230A} allele). Values were plotted as percentage of WT values after normalization against the telomere region (TEL) amplified using a telomere-specific primer pair (Table 3). Error bars represent values obtained from two independent experiment using three biological replicates.
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ACS1p, and ADY2p) in the absence of CAT8, and the impairment was rescued in the cat8Δ strain expressing ADR1. This result suggests that Bmh binding to Adr1 influences Swi/Snf recruitment. In summary, Bmh appears to inhibit Swi/Snf but not Mediator recruitment, and both are important for coactivated transcription, suggesting that Mediator function might be impaired by the presence of Bmh in the absence of Cat8.

**DISCUSSION**

The pol II transcription cycle comprises of three basic steps: 1) initiation, including the recruitment of general transcription factors and hypophosphorylated pol II to the promoter forming the PIC; 2) elongation, including the clearance of pol II from the promoter-bound factors (early elongation complex), promoter-proximal pausing, and elongation along the gene body; and 3) termination, including nascent transcript cleavage and polyadenylation. Transcription can be regulated at any one of these stages (55). In principle, combinatorial regulation could be achieved in all three basics step of a transcription cycle.

Our results demonstrate that Bmh binding of Adr1 prevents PIC formation or function unless the co-regulatory TF, either Cat8 or Oaf1/Pip2, is present. This mode of regulation of transcription by 14-3-3 proteins has not been observed previously. In the systems studied previously, 14-3-3 influences transcription by blocking DNA binding and nuclear localization signal(s) (e.g. Forkhead transcription factor FOXO), by inducing the structural organization of a transcription factor (e.g. p53 and HDAC5), and by anchoring a protein in the cytoplasm (e.g. histone deacetylase) (reviewed in Ref. 56). Our previous studies have demonstrated that yeast 14-3-3 (Bmh) regulates Adr1- and Cat8-activated combinatorial transcription by inhibiting the activity of Adr1 through direct binding to the latter (26, 27).

Our current study suggests that PIC formation is inhibited by Bmh. This conclusion is based on ChIP assays of the abundance of five different PIC proteins: pol II, TBP, TFIIIB, THIF, and TFIIH, at several Adr1-dependent promoters. All five of these proteins/protein complexes were readily detected in the absence but not the presence of Bmh activity in a cat8Δ mutant. In addition, PIC assembly directed by the Adr1AD, but not by two heterologous ADs or by artificially recruited Mediator, was inhibited by Bmh. The weak H3K4me3 signal in the absence of Cat8 confirms the absence of an elongating RNA pol II complex, in agreement with the low abundance of RNA pol II and multiple general TFs. We interpret these results to indicate that the presence of the second activator, Cat8 in this case and Oaf1/ Pip2 in the case of β-oxidation and peroxisomal genes, suppresses inhibition by Bmh by an unknown mechanism.

The data supporting a role for Bmh in inhibiting PIC formation is in apparent conflict with our previously reported data suggesting that Bmh inhibits PIC function but not assembly (26). We confirmed our previous results, which were based on ChIP analysis, using the well studied 8WG16 antibody. This antibody recognizes unphosphorylated and pSer-5-CTD but not pSer-2-CTD (57). The relative ChIP signal at the three promoters assayed was lower for the 8WG16 antibody than the other six antibodies employed, but the reproducibility in several different experiments, in multiple strains and genetic back-grounds, argues against an artifactual origin for the evidence for RNA pol II recruitment in the absence of Cat8 when Bmh is present. On the other hand, the data supporting a role for Bmh in PIC formation was stronger, as it is based on the absence of a ChIP signal using six different antisera (anti-Rpb3, anti-pSer-5-CTD, anti-TBP, anti-TFIIB, anti-Tfg2, and anti-Ssl2) when Bmh was present and Cat8 was absent (Fig. 7). Moreover, recruitment of RNA pol II, as judged by ChIP for Rpb3, was also regulated by Bmh at GAL promoters driven by GBD fusion proteins with and without the Adr1 RD (Fig. 6). One possible explanation for this apparent conundrum invokes several changes in PIC composition or structure that might be inhibited by Bmh in the absence of the second TF. For example, the availability of the Rpb3 epitope might be masked in some unknown manner when Bmh is present without Cat8. Furthermore, Bmh might inhibit the recruitment of general TFs. The low ChIP signal with the pSer-5-CTD antibody in a strain containing GBD-Adr1AD and in a cat8Δ strain with WT Adr1 (Fig. 7, C and D) could indicate that the RNA pol II that was detected by the 8WG16 antibody was non-phosphorylated, an interpretation that is consistent with a lack of transcription initiation. The nature of the PIC assembled in the presence of Bmh when Adr1 is present and the co-regulatory TF is absent needs to be analyzed further to resolve the current uncertainty. The ambiguity highlights the need for an unbiased method of analyzing PIC composition. Regardless of how PIC function is affected, whether by altered assembly or altered function, our data show that initiation is the step in the transcription cycle that is inhibited by Bmh to ensure a synergistic and regulated response to glucose depletion and fatty acid induction.

PIC formation does not appear to be regulated by histone H3 or H4 acetylation, because we observed comparable levels of these modifications in the presence and absence of either Bmh or Cat8 function or Bmh binding region (RD) mutations in Adr1. Although we observed a strong stimulation of total histone acetylation at Adr1-dependent promoters after glucose depletion (14) (Fig 10A), the abundance of these modifications was similar in the presence and absence of Bmh in the cat8Δ mutant. These results are consistent with the observation that GAL mRNA abundance in the presence of GBD-RD-Adr1AD was only 2-fold dependent on Gcn5 (Fig 11A). These observations are also consistent with our previous data showing similar amounts of two histone acetyltransferase complexes, SAGA and NuA4, at Adr1-dependent promoters in the absence of Cat8 (22) and with the interpretation that activators such as Adr1 recruit coactivators but not general transcription factors or RNA pol II.

Our results suggest that Bmh regulates Adr1 activity and thus combinatorial transcription via Mediator and Swi/Snf, because we observed significant disruption of transcription in the absence of Snf2 and Gal11 only when the Adr1AD, not a Bmh-insensitive hAD, was fused to GBD-RD. Surprisingly we did not detect any recruitment defect in the Mediator complex at the Adr1-dependent promoters when Bmh was present, suggesting that Bmh might impair the function of Mediator in an activation domain-specific manner. However, Mediator artificially recruited to a promoter by fusion of Gal11 to either GBD or Adr1DBD was not subject to Bmh-mediated inhibition, sug-
suggesting that the mechanism of Mediator recruitment could play a role in Bmh inhibition. Alternatively, the Gal11 fusion protein might alter the structure of Mediator and render it insensitive to Bmh-mediated inhibition. We observed an ~40–60% reduction in Swi/Snf recruitment due to Bmh-mediated inhibition when Cat8 was absent, suggesting that Bmh might inhibit PIC formation or function in part by regulating Swi/Snf recruitment and consequent Swi/Snf-dependent chromatin remodeling. We observed a comparable level of H3KAc in the presence and absence of CAT8, suggesting that histone acetylation could occur independently of chromatin remodeling by the Swi/Snf complex, which is consistent with our previous observation that the absence of a functional Swi/Snf complex had less than a 2-fold effect on Gcn5 recruitment at Adr1-dependent promoters (52). Regulation of transcription by Bmh via Mediator and Swi/Snf was further supported by the observation that ADRI° alleles (30) could partially bypass the requirement for these coactivators (16).

How Bmh regulates a step occurring after Mediator recruitment is unknown. One possibility is that recruitment of Swi/Snf is regulated by Mediator, as we observed a 2-fold reduction in Snf2 recruitment at the ADH2 promoter in a mediated strain, but Mediator recruitment was unaffected in a snf2 mutant (52). However, the 2-fold reduction of Swi/Snf recruitment does not explain the 10–50-fold reduction in mRNA abundance. One possible mechanism is through an allosteric change in Mediator that regulates PIC formation or function. Transcriptional activation through the structural modulation of Mediator via an activator has been reported when p53 was the activator has been reported when Adr1 was the activator (17). We hypothesize that Bmh might inhibit Mediator activity through a structural modulation that is achieved through binding to Adr1.

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