Snf1 Protein Kinase Regulates Adr1 Binding to Chromatin but Not Transcription Activation*

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The yeast transcriptional activator Adr1 controls the expression of genes required for ethanol, glycerol, and fatty acid utilization. We show that Adr1 acts directly on the promoters of ADH2, ACS1, GUT1, CTA1, and POT1 using chromatin immunoprecipitation assays. The yeast homolog of the AMP-activated protein kinase, Snf1, promotes Adr1 chromatin binding in the absence of glucose, and the protein phosphatase complex, Glc7-Reg1, represses its binding in the presence of glucose. A post-translational process is implicated in the regulation of Adr1 binding activity. Chromatin binding by Adr1 is not the only step in ADH2 transcription that is regulated by glucose repression. Adr1 can bind to chromatin in repressed conditions in the presence of hyperacetylated histones. To study steps subsequent to promoter binding we utilized miniAdr1 transcription factors to characterize Adr1-dependent transcription in vitro. Yeast nuclear extracts prepared from glucose-repressed and glucose derepressed cells are equally capable of supporting miniAdr1-dependent transcription and pre-initiation complex formation. Nuclear extracts prepared from a snf1 mutant support miniAdr1-dependent transcription but are partially defective in the formation of pre-initiation complexes with Mediator components being particularly depleted. We conclude that Snf1 regulates Adr1-dependent transcription primarily at the level of chromatin binding.

The absence of a fermentable carbon source signals yeast nuclei to activate expression of genes controlling aerobic metabolism of alternative carbon sources (1–3). Numerous transcription factors allow the cell to generate energy and metabolites from non-glucose carbon sources such as ethanol and glycerol, as well as from storage carbohydrates and lipids. Many of these transcription factors are controlled positively by Snf1, a yeast homolog of AMP-activated protein kinase, and negatively by Glc7, a type-1 protein phosphatase, and one of its regulatory subunits, Reg1 (4). Integration of the metabolic pathways utilized in the absence of glucose is mediated in part through combinatorial interaction of transcription factors that regulate genes acting in more than one pathway. Adr1 is an example of such transcription factor. Adr1 was discovered by virtue of its activation of ADH2, encoding the ADHII isozyme responsible for the first step in ethanol utilization (5). Adr1 synergistically activates expression of ADH2 and ACS1, encoding acetyl-CoA synthetase, by binding to UAS1 in combination with a second glucose-regulated transcription factor required for two-carbon metabolism, Cat8. Cat8 binds to the adjacent UAS2/CSRE in the ADH2 and ACS1 promoters (6–11). Adr1 is also thought to act in concert with the olate-regulated transcription factor Oaf1/Pip2 to activate expression of genes such as CTA1, SPS19, POX1, PEX11, and POT1, which are involved in β-oxidation of fatty acids and peroxisome biogenesis (12–16). A third example of this combinatorial control may occur at the GUT1 promoter, where both Adr1 and Ino2/Ino4 play important roles in derepression (17). However, evidence of the in vivo binding of Adr1 to the promoters of these genes exists only for ADH2 (18).

Adr1 is a large transcription factor containing a complex DNA binding domain consisting of C2H2 zinc fingers and a proximal accessory region (PAR). A nuclear localization signal is located near the amino terminus (19), and four transcription activation domains (TADs) have been identified by deletions and gene fusions to LexA (20) and GAL4 (21). TADI (amino acids 1–220) is in the DNA binding amino terminus. TADII is contained within amino acids 263–359. TADIII, present within amino acids 420–462, is the best characterized of the four TADs and is the only Adr1 TAD that is active when fused to both LexA and Gal4. TADIII contains several copies of the conserved activation motif FXXφΦφ that are important for its function. TADIV is located between amino acids 642 and 704. In vitro binding studies as well as in vivo co-immunoprecipitation suggest that these TADs interact with TFIIB, TFID, Ad2a, and Gen5 (22, 23). Thus, Adr1 may play an important role in recruiting an active pol II complex to promoters. In addition, Adr1 plays an essential role in mediating chromatin remodeling at the ADH2 promoter prior to derepression (24, 25), and TADIII is sufficient for this remodeling (26).

Regulation of Adr1 is complex, possibly involving transcriptional, translational, and post-translational processes (27–32). BCY1, encoding the regulatory subunit of cAMP-dependent protein kinase is a positive regulator of ADR1 expression (33), and the catalytic subunits of the kinase phosphorylate Ser-230 of Adr1 in vivo and in vitro (28, 29). However, phosphorylation of Ser-230 in vivo appears to modulate the transcriptional activity of Adr1 rather than alter its sensitivity to glucose repression (29, 31, 33). The signals acting upstream of BCY1 in

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† The abbreviations used are: Snf1, proteins are indicated by gene names with a large first letter followed by small letters; SNF1, genotypes and gene names are capitalized in italics; ABC, Adr1 DNA binding domain, amino acids 17–165; TAD, transcription activation domain; TBP, TATA binding protein; TF, TBP-associated factor; PAR, proximal accessory region (of Adr1); ADH, alcohol dehydrogenase; PIC, pre-initiation complex; ChIP, chromatin immunoprecipitation; UAS, upstream activation sequence; WT, wild-type; pol, polymerase; SM, synthetic medium; HA, hemagglutinin; CSRE, carbon source response element.

2 E. T. Young and K. M. Dombek, unpublished observations.
Glucose regulation acts through the Adr1 DNA binding domain (ABD, amino acids 1–165). A protein fusion containing ABD and either the VP16 TAD or TADIII is able to confer glucose-regulated expression on ADH2 (31). A gene fusion encoding nearly the entire Adr1 open reading frame (amino acids 17–1323), and the DNA binding domain of Gal4 is glucose-regulated at UAS1-containing but not at UASG-containing promoters (31), suggesting that glucose repression mediated by ABD is promoter-specific. Consistent with this interpretation, chromatin immunoprecipitation (ChIP) studies demonstrated that Adr1 binds to the ADH2 promoter only after glucose has been depleted from the medium (18). The nuclear localization of Adr1 is not regulated, suggesting that Adr1 is within the nucleus in the presence of glucose but is unable to bind ADH2 chromatin (31). Adr1 isolated from repressed and derepressed cells is equally competent to bind DNA (32). Taken together the results suggest that the access of Adr1 to DNA may be restricted by chromatin, even though UAS1 appears to be in a nucleosome-free region (24). Whether regulation acts directly through Adr1, for example by modifying the chromatin binding activity of Adr1, or through modification of chromatin to restrict access of Adr1 to the promoter, or through some combination of these mechanisms is not known.

Recent studies suggest that binding of Adr1 to the ADH2 promoter in repressed conditions is not sufficient for activation of transcription (18). Hyperacetylation of the ADH2 promoter caused by mutations in the histone deacetylases RPD3 and HDAl allows Adr1 to bind the ADH2 promoter in glucose-repressed conditions, but recruitment of TBP does not occur. The latter observation suggests that a second step in transcription initiation after binding of Adr1 is regulated by glucose repression. One possibility is that one or more of the TADs confer an additional level of regulation that is required for TBP recruitment.

The present studies show that the ability of Adr1 to bind the ADH2, ACS1, POT1, CTA1, and GUT1 promoters in vitro is glucose-regulated by Snf1 and Reg1. To study the possible regulation of activation domain function, we employed an Adr1-dependent nuclear extract transcription assay. In this assay a simplified version of Adr1, called miniAdr1, is used as the transcription activator. miniAdr1 contains ABD fused to a single activation domain, TADIII. The nuclear extract supplemented with recombinant miniAdr1 and an appropriate DNA template has many of the transcriptional properties displayed by Adr1 in vivo. However, recombinant miniAdr1 is active in nuclear extracts prepared from glucose-repressed cultures, and is Snf1-independent, suggesting that Snf1 functions only to facilitate chromatin binding by Adr1 and not to enhance its activation potential.

### EXPERIMENTAL PROCEDURES

**Strains**—Yeast strains are listed in Table I. *Escherichia coli* strains used for plasmid propagation are DH5α and XL1. *Escherichia coli* MC1061 (pREP) is used for preparation of recombinant miniAdr1 proteins.

**Growth of Yeast Cultures**—Yeast strains were grown in YPD or synthetic medium (SM) prepared according to standard methods (40). Glucose-repressed cultures were started in YP or supplemented SM containing 5% glucose. Derepressing cultures were inoculated into YP or supplemented SM containing 0.05% glucose and 3% ethanol when indicated. Transformation of yeast used a modified LiAcetate protocol (41).

**Plasmid Construction**—The miniAdr1 activator and reporter plasmids are similar to those described (see Refs. 21, 31, 42, and 43). miniAdr1 has a 2-amino acid spacer between ABD and TADIII. miniAdr1 ends at amino acid 160 of finger two, and the two copies of finger one are separated by TNEKPY, the linker amino acids present between finger one and finger two. It has a His6 tag at its amino terminus for purification. The miniAdr1 genes are transcribed under the control of the native ADR1 promoter on a centromere-containing plasmid. The reporter plasmid containing binding sites for miniAdr1 in the HIS4 promoter was constructed from pSH515 obtained from S. Hahn (44) by inserting the double-stranded oligonucleotide SK half (42) into pREPOI. This introduced a single Adr1 binding site into the chromatin. Adr1 isolated from repressed and derepressed cells is equally competent to bind DNA (32). Taken together the results suggest that the access of Adr1 to DNA may be restricted by chromatin, even though UAS1 appears to be in a nucleosome-free region (24). Whether regulation acts directly through Adr1, for example by modifying the chromatin binding activity of Adr1, or through modification of chromatin to restrict access of Adr1 to the promoter, or through some combination of these mechanisms is not known.

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activity assays were performed as described previously (35).

Chromatin-immunoprecipitation Assays—Cells in 100 ml of culture were treated for 5 min with 1% formaldehyde at 25 °C. The crosslinking was stopped by addition of glycine to 0.125 M, and the cells were washed and processed for immunoprecipitation as described previously (46). Immunoprecipitation of HA-tagged Adr1 bound to chromatin used rabbit polyclonal anti-HA antisera (F-7, Santa Cruz Biotechnology) and protein A-Sepharose beads. Oligonucleotides for PCR amplification are listed in Table II. Real-time quantitative PCR was performed on an Applied Biosystems ABI7700 and analyzed using software provided by the manufacturer. The slope of the curve of input DNA versus PCR cycles was used to calculate the DNA concentration of the PCR products generated by ACT1 and ADH2 primers using DNA immunoprecipitated using anti-HA antisera. The amount of ADH2 product in each sample was corrected for the amount of ACT1 product formed using the same immunoprecipitated DNA. The ACT1 DNA is nonspecifically immunoprecipitated by the antisera. This contamination serves as an internal control for the PCR efficiency in each experimental DNA sample.

Nuclear Transcription Extracts and Assays—Nuclear extracts were prepared and used as described (44, 47) from yeast spheroplasts prereplicated by the antisera. This contamination serves as an internal control for the PCR efficiency in each experimental DNA sample. A primer extension assay was performed using a primer extending from the polymerase initiation codon of the open reading frame.

RESULTS

Adr1-dependent Derepression Is Regulated by Binding to Chromatin—We used a HA-epitope-tagged version of Adr1 in a strain containing three extra chromosomal copies of ADR1 (31) to study glucose-regulated chromatin binding. In this strain derepression of ADH2 expression occurs more rapidly than in the presence of a single copy of ADR1, but ADH2 expression remains stringently glucose repressed (31) and Snf1-dependent.3 Fig. 1A shows that in the multicyclic Adr1 strain chromatin binding occurs rapidly after glucose depletion. Fig. 1B indicates that re-addition of glucose leads to an equally rapid loss of binding by Adr1. Real-time quantitative PCR analysis was used to quantify binding of Adr1-HA to the ADH2 promoter. The quantitative data are shown underneath the lanes in Fig. 1. Using this assay, binding is detected 5 min after glucose depletion (Fig. 1A) and increases 100-fold by 30 min. Within this time interval there is no change in the level of Adr1 (31), suggesting that chromatin binding by Adr1, and loss of binding activity when glucose is added, are regulated at the post-translational level. This interpretation is confirmed by the data in Fig. 1C showing that Adr1 binds to chromatin when the cells are derepressed in the absence of protein synthesis. Unexpectedly, a low level of Adr1 binding to chromatin is detected in the presence of glucose and cycloheximide. In the presence of cycloheximide and glucose, a low level of ADH2 mRNA was observed that prompted us to suggest that ADH2 mRNA might be synthesized at a low rate in repressed conditions and stabilized in the absence of protein synthesis (31). The observation that chromatin binding by Adr1 is enhanced during cycloheximide treatment of glucose-repressed cells makes it more likely that ADH2 transcription, and by inference, Adr1 modification, is directly affected by the absence of protein synthesis.

ADH2 is dependent on Snf1 protein kinase for derepression (5, 33, 36). To determine whether Snf1 is required for chromatin binding, we performed ChIP of the ADH2 promoter in a strain lacking SBF1. Adr1 binding was not detected, and real-time quantitative PCR indicated that the level of binding was reduced at least 20-fold in the absence of SBF1 (Fig. 1D). Thus, Snf1 is essential for chromatin binding by Adr1.

The Reg1-Glc7 protein phosphatase is necessary for glucose repression of ADH2 expression (34, 35). The reg1-dependent constitutive expression is dependent on Adr1, indicating that reduced Glc7 activity must allow Adr1 to bind the promoter in repressed conditions. Activation of ADH2 expression in a reg1 or a glc7 mutant in glucose-growth conditions is incomplete, suggesting that one or more GLC7- and REG1-independent pathways contributes to ADH2 repression. To determine whether this putative REG1-GLC7-independent pathway acts before or after chromatin binding, we examined Adr1 binding

| Gene | Name | Position/size | Sequence |
|------|------|--------------|----------|
| ADH2 | MD50 | 431 to 415 | CATTATGGATTCCGCTCCCG |
| ADH2 | JS-2 | 429 to 415 | GTGACCCCGAATTTCCTT |
| ADH2 | ADH2-Q1 | 428 to 415 | ACCATCCCTCCAGAAGCTT |
| ADH2 | ADH2-Q2 | 427 to 415 | TCAATGCTCAAACGGAATTCCTTA |
| ACT1 | SK-half | 426 to 415 | GTGACCTAGAAGTGGAATGAGTCCCGG |
| ACT1 | ACT1-A | 425 to 415 | GACCAAGCATCCAGATG |
| ACT1 | ACT1-B | 424 to 415 | ACCAGGTGAGTAAACCCATTACCCC |
| ACT1 | ACT1-C | 423 to 415 | ATGCTCCCGAATTGGCAGAAT |
| ACT1 | ACT1-D | 422 to 415 | CCAAGATGACTGCTGAG |
| ACT1 | ACT1-E | 421 to 415 | TATTGGCAACCAGAAGACTG |
| ACS1 | ACS1-B | 420 to 415 | GAGTTGTGGCCGGAACTAT |
| ACS1 | ACS1-C | 419 to 415 | GGACATTACAGTGAAGAATGCT |
| ACS1 | ACS1-D | 418 to 415 | GGAATTAGCCGCGCAAGT |
| ACS1 | ACS1-E | 417 to 415 | CACTCACCTGTTGACATGCC |
| ACS1 | ACS1-F | 416 to 415 | AAGATGGAAGGACGGAAAGC |
| ACS1 | ACS1-G | 415 to 415 | TATTTTGCAACCAGAAGACTG |
| CTA1 | CTA1-A | 414 to 415 | GGAAATTAGCCGCGCAAGT |
| CTA1 | CTA1-B | 413 to 415 | GGACATTACAGTGAAGAATGCT |
| GUT1 | GUT1-A | 412 to 415 | GGATTGTCCCGGAACTAG |
| GUT1 | GUT1-B | 411 to 415 | TTGGCAATTGCGAGATCC |
| POT1 | POT1-A | 410 to 415 | GAAATGGAAGGACGGAAAGC |
| POT1 | POT1-B | 409 to 415 | GAGCAACTAAGAAATTAACCATCAGAT |

* Position is with respect to the ATG initiation codon of the open reading frame.

3 E. T. Young, unpublished observations.
in a reg1 mutant. If Adr1 occupies the ADH2 promoter at the same level in a reg1 mutant grown in repressed conditions as it does in derepressed conditions in a wild-type strain, then the putative REG1-GLC7-independent pathway must operate after Adr1 has bound the promoter. Alternatively, if the REG1-GLC7-independent pathway contributes to the ability of Adr1 to bind the promoter, we would expect to see reduced binding of Adr1 to the promoter in a reg1 mutant strain grown in repressed conditions. Real-time quantitative PCR detected Adr1 binding significantly above the background level in a reg1 mutant, but chromatin binding to the ADH2 promoter is still 30-fold below the level observed after derepression (3.2 units relative to 100 units for fully derepressed wild-type cells), consistent with the relative levels of ADH2 expression in wild-type derepressed versus reg1 mutant repressed cultures. Adr1 levels are elevated in a reg1 strain (35) but not to the level observed in strains containing multiple copies of Adr1 (31). Because no Adr1 is detected bound to chromatin in the latter strain (Fig. 1A), the modest elevation in Adr1 level in the reg1 strain cannot explain chromatin binding. Thus, Adr1 binding to chromatin is regulated by Glc7:Reg1 and by a second pathway that is independent of REG1-GLC7. This putative second pathway is Snf1-dependent, because no binding is detected in a snf1 mutant.

To determine the generality of glucose-regulated chromatin binding by Adr1, we performed ChIP analysis of four other ADR1-dependent genes representing three metabolic pathways: ACS1 (acyetyl-CoA synthetase), POT1 (peroxisomal-CoA thiolase), CTA1 (peroxisomal catalase), and GUT1 (glycerol kinase). Adr1 directly regulates the expression of these genes by binding to the promoters only in derepressed conditions (Fig. 1D). Thus, regulation at the level of chromatin binding is a common property of ADR1-dependent genes.

Characterization of miniAdr1—We used miniAdr1, a small, functional version of Adr1 (31) to develop an in vitro transcription system. miniAdr1 contains the DNA binding domain and nuclear localization signal (ABD, amino acids 1–172), and a transcription activation domain, TAD III (amino acids 420–462) from Adr1. One advantage of miniAdr1 is its small size, which allows usable quantities of recombinant protein to be purified intact from E. coli.

ADH2 expression dependent on miniAdr1 is stringently glucose-regulated, suggesting that it retains regulatory targets of Snf1 and Reg1:Glc7 (31). To demonstrate this directly we transformed adr1-null strains that were wild-type or mutant for SNF1 or REG1 with a miniAdr1 expression plasmid and assayed ADH2 expression. ADH2 derepression was Snf1-dependent, and constitutive ADH2 expression was observed in a reg1 mutant.3 Thus, miniAdr1 appears to be regulated like the full-length Adr1 protein.

Development of a miniAdr1-dependent in Vitro Transcription System—miniAdr1 purified from E. coli was tested in an unfraccionated nuclear extract transcription system using a primer extension assay to measure transcription. The nuclear extract transcription system contains all of the components needed for transcription by the pol II holoenzyme and is capable of multiround, activator-, TAF-, and mediator-dependent transcription (44, 47). miniAdr1 is active in this system using a template containing multiple UAS1 elements (10 binding sites). The RNA product initiates at the correct site based on its position relative to a-amamin.3

However, the activity of miniAdr1 in the nuclear transcription extract is low compared with a Gal4-VP16 activator, and it is inactive using a template containing a single Adr1 consensus binding site, or two perfect binding sites in inverted orientation, UAS1, the preferred sequence in vivo (43). The relatively low DNA binding affinity of Adr1 compared with Gal4 (Kd of 10^{-7} versus 10^{-9}, respectively (42)) could explain its low activity in vitro. miniAdr1 also has low DNA binding specificity, a
Chromatin Binding and in Vitro Transcription by Adr1

Table III
mini3Adr1-dependent gene expression is glucose-repressed, dependent on Snf1 for derepression, and requires a TATA box

| Activator | Reporter | β-Galactosidase activity |
|-----------|----------|-------------------------|
| Snf1       |          | Repressed | Derepressed | Repressed | Derepressed |
| ABD       | HIS4-TATA-lacZ  | 1.0        | 2.0         | 1.0        | 1.0         |
| Mini3Adr1 | HIS4-TATA-lacZ  | 14         | 280         | 8          | 16          |
| ABD      | HIS4-GAGA-lacZ  | <1         | 1.1         | 1.0        | 1.0         |
| Mini3Adr1 | HIS4-GAGA-lacZ  | <1         | 1.1         | 1.0        | 1.0         |
| ABD      | CYC1-TATA-lacZ  | 2.0        | 17          | 5.0        | 4.0         |
| Mini3Adr1 | CYC1-TATA-lacZ  | 20         | 160         | 22         | 18          |

Strains TYY 202 and 390, SNF1 and snf1A::URA3, respectively, containing the indicated plasmids, were grown in selective media (trp - ura - )) plus 5% glucose (Repessed) to about 2 × 10^6 cells/ml. A portion of the culture was assayed for β-galactosidase activity as described under "Experimental Procedures," and cells from another portion were collected by centrifugation and resuspended in selective media plus 0.05% glucose (Derepressed) and grown for 18 h at 30 °C. A portion of the culture was removed and assayed for β-galactosidase activity. The β-galactosidase activities are Miller units. Activator plasmids are YCpmi3Adr1Δ172 (ABD) and YCpmi3Adr1 reporter plasmids are pNK 101 and pNK 102 (YeplAS1-3Fonsensus/HIS4/lacZ and YeplAS1-3Fonsensus/CYC1/lacZ, respectively.

To test the dependence of mini3Adr1 on TADIII, we performed transcription in vitro using mini3Adr1 lacking TADIII or containing mutated versions of TADIII. The HA2 activator contains mutations in two activation motifs (21). Activator CR9 has mutations that reduce the negative charge of the acidic patch at the carboxyl terminus of TADIII. Both mutants are weak activators in vivo (21). No activation could be detected above the basal level in the absence of TADIII (Fig. 2C, ABD).

Mutations in the DNA binding domain also reduce in vitro transcriptional activity. The mutations tested alter the DNA binding domain by deletion of PAR (42, 48) from miniAdr1 containing either two or three fingers. The two-finger miniAdr1 used in these experiments has a change-of-specificity mutation in the second finger, Leu-146 to His, that enhances DNA binding about 70-fold (42). miniAdr1 (L146H, F1H in Fig. 2D) is a weaker activator than miniAdr1 (3F), despite its higher binding affinity. Deleting PAR (∆N) reduces the activity of both miniactivators.

In summary, mini3Adr1 behaves in most respects as a classic activator in vitro: it requires a high affinity DNA binding domain and a strong activation domain outside of the DNA binding domain. Transcription requires a TATA sequence in the promoter, implying a dependence on TBP for initiation. An importance difference between mini3Adr1 and most other eukaryotic transcription activators is the requirement for a region of the protein within the DNA binding domain, the PAR region, for maximal transcriptional activation both in vivo and in vitro.

Recombinant mini3Adr1 Is Active in Nuclear Transcription Extracts Prepared from Repressed Cells—The initial in vitro transcription experiments were performed using nuclear extracts prepared from glucose-grown cells. Because mini3Adr1 is most active in vivo in cells depleted of glucose, we prepared and tested nuclear extracts from both repressed and derepressed cells. As shown in Fig. 3, transcription extracts prepared from derepressed cells are as active as those prepared from repressed cells using mini3Adr1 as activator. The activity of the transcription extracts prepared from repressed cells was tested at several protein concentrations with the same results. Thus, repression of recombinant mini3Adr1 activity is not observed in nuclear transcription extracts prepared from glucose-repressed yeast cells.

Recombinant mini3Adr1 Is Active in Nuclear Extrasts Prepared from snf1 Mutant Cells—Because mini3Adr1 is unable to activate transcription in a snf1 mutant (Table III), mini3Adr1

measure of its specific to nonspecific DNA binding affinity (42). Mini3Adr1 contains an additional zinc finger is glucose-repressed—if low DNA binding affinity and low specificity cause reduced transcriptional activity of miniAdr1 in vitro, we reasoned that addition of a third finger might enhance its activity as a transcription factor in vitro. We made and tested a modified version of miniAdr1 containing an additional finger (mini3Adr1) with the same activation domain (TADIII). A related three-finger Adr1 protein binds its specific DNA site with about 20-fold higher affinity and has a 5-fold higher specificity than miniAdr1 containing two fingers (42).

To ensure that mini3Adr1 is regulated in the same manner as wild-type Adr1, we tested its activity in vivo by expressing it from the ADR1 promoter on a low copy number, centromere-containing plasmid. A reporter gene was constructed by inserting a single consensus-binding site, GGCGGGTGT, for mini3Adr1 upstream of two promoter fusions, CYC1/lacZ and HIS4/lacZ. The former is the promoter used for previous reporter studies with miniAdr1 (42, 43). The latter is a modified HIS4 promoter that is identical to the promoter used as the template for the in vitro transcription studies. After transformation of appropriate yeast strains with activator and reporter plasmids, β-galactosidase activity was measured to assess the regulation of mini3Adr1. The data in Table III show that both promoters are glucose repressed and mini3Adr1-dependent for derepression. The HIS4 promoter is more tightly repressed (20-fold) than the CYC1 promoter (8-fold) is. Although UAS1 (3F)/HIS4/lacZ is strongly glucose-repressed, its activity on glucose is stimulated 14-fold by mini3Adr1, suggesting that mini3Adr1 is able to activate transcription weakly in repressed conditions. Table III shows that mini3Adr1-dependent transcription in vivo is dependent on the HIS4 TATA sequence, because mutation to GAGA abolished activity. Table III also shows that mini3Adr1 is SNF1-dependent. Thus, mini3Adr1 displays the regulated properties of intact Adr1 and is an appropriate activator to use for in vitro studies.

Recombinant mini3Adr1 activates transcription in vitro and depends on TBP, TADIII, and high affinity DNA binding—When recombinant mini3Adr1 is added to a nuclear extract transcription system containing a template with a single optimal binding site, a transcript of the appropriate size is observed (90 nucleotides, Fig. 2A). In the experiment shown mini3Adr1 had lower activity than Gal4-VP16. In other experiments their relative activities were more similar. Both basal and mini3Adr1-dependent transcription is dependent on the HIS4 TATA box, indicating that TBP is required for miniAdr1-dependent transcription in vitro and that the template has a single functional TATA sequence (Fig. 2B).

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activity in snf1 nuclear transcription extracts was tested. Fig. 4 shows that nuclear extracts isolated from a snf1 mutant support mini3Adr1-dependent transcription as well as wild-type nuclear extracts, independent of growth conditions prior to making the extracts (low or high glucose). The level of basal transcription is also similar in all four extracts. No reproducible difference was detected as long as extracts that were prepared from cells grown in high and low glucose. No reproducible difference was detected between nuclear transcription extracts isolated from a snf1 mutant and the wild type. A similar result was obtained by using the wild-type strain TYY202 grown in YPD containing 5% glucose (R) or shifted to YPD-0.05% glucose (DR) for 6 h. Basal and mini3Adr1-dependent transcription were assayed by primer extension assays. –, no activator; +, mini3Adr1; +1, expected transcription initiation site; MW, molecular weight markers in nucleotides.

Formation of PICs is dependent on intact mini3Adr1. This is most clear for the recruitment of TFIIB, TOA2, and members of the Mediator and SAGA complexes: Med6, Srb2, Srb4, Srbl0, and Gcn5, all of which show a 2- to 10-fold dependence on mini3Adr1 for PIC formation (Fig. 5). As has been observed previously (44, 47), some components of the PIC show less activator dependence than do others, presumably because they are bound more tightly and perhaps nonspecifically to DNA in the absence of activator.

In the absence of TADIII (ABD alone) PIC formation is reduced to a level lower than that observed in the absence of activator (Fig. 5). The apparent inhibition of PIC formation suggests that ABD may have a negative effect on PIC formation in the absence of a transcription activation domain. These data are consistent with the transcription studies that failed to detect a transcription activation function associated with the DNA binding domain alone. In parallel with its reduced activity in the transcription assay and in vivo, the TADIII-HA2 mutant reduced PIC formation 2- to 5-fold.

Surprisingly, PIC formation was not significantly affected by mutation of the TATA box (Fig. 5). The same mutation reduced transcription in vitro (Fig. 2B) and in vivo (Table III) to very low levels. Thus, mini3Adr1 is able to form PICs in the absence of a TATA sequence, but the TATA box is nevertheless required for PIC formation (Fig. 5). As has been observed previously (44, 47), some components of the PIC show less activator dependence than do others, presumably because they are bound more tightly and perhaps nonspecifically to DNA in the absence of activator.

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snf1 Mutant Nuclear Extracts Are Partially Defective in PIC Formation—We analyzed PIC formation in nuclear extracts prepared from cells grown in high and low glucose. No reproducible difference was detected as long as extracts that were active for in vitro transcription were compared. However, we detect a lower level of PIC formation using a snf1 nuclear transcription extract (Fig. 6). This is particularly true using an extract prepared from derepressed snf1 mutant cells. A similar
A lower level of PIC formation might be observed if some of the PIC components are present at reduced levels in the nuclear extracts. This explanation seems unlikely because the transcriptional activities of the wild-type and snf1 extracts are similar. To test this possibility directly, we analyzed the nuclear extracts by Western blotting for several of the components that were analyzed in the PICs. No significant differences in the total levels of the proteins were observed in the nuclear extracts. These results suggest that PIC formation on the mini3Adr1-dependent promoter is reduced in rate or extent when the nuclear transcription extract is prepared from a snf1 mutant. The relative insensitivity of the transcription assay to this deficiency could be due to different templates being used (plasmid DNA versus a DNA fragment attached to a bead) or to different steps being rate-limiting in the two assays.

**FIG. 5.** mini3Adr1-dependent pre-initiation complex (PIC) formation is independent of a TATA sequence in the promoter but requires TADIII. PIC formation was assayed on DNA templates immobilized on magnetic beads as described under “Experimental Procedures.” TATA, wild-type promoter template; GAGA, mutant promoter template in which the HIS4 TATA sequence is changed to GAGA (44); WT, wild-type mini3Adr1 activator; HA2, mutant mini3Adr1 activator containing mutations in TADIII (21); ABD, mini3Adr1 lacking TADIII. The Western blot was probed sequentially for the indicated proteins as well as for mini3Adr1 (not shown). mini3Adr1 was present at similar levels in all of the supplemented reactions.

**FIG. 6.** Pre-initiation complex formation is reduced in snf1 mutant extracts. Nuclear transcription extracts were prepared from wild-type SNF1 and snf1 mutant strains, TYY202 and TYY390, respectively, grown in derepressed conditions (see legend to Fig. 4). mini3Adr1 was used to direct PIC formation on DNA templates immobilized on magnetic beads as described under “Experimental Procedures.” After polyacrylamide gel electrophoresis and Western blotting to identify specific proteins, antibody signals were quantified by fluorometry. The intensity of the signal for each protein was normalized to the value observed for basal transcription in wild-type SNF1 extracts. A, example of a Western blot probed for Rpb1, Rpb3, and mini3Adr1. B, quantification of antibody signals for: I, Swi3; 2, Srb2; 3, Srb4; 4, Med6; 5, TBP; 6, TFIIB; 7, TOA2; 8, Rpb1 on a scale normalized to the amount of each protein present in SNF1 wild-type PICs formed in the absence of activator mini3Adr1.

**DISCUSSION**

Activation of Adr1-dependent genes appears to be regulated by glucose repression at two steps prior to initiation of transcription. The first step is DNA binding. Five Adr1-dependent promoters, ADH2, CTA1, ACS1, GUT1, and POT1, are bound by Adr1 in vivo only when glucose is absent from the media. Regulated Adr1 binding to the ADH2 promoter requires Snf1 protein kinase and is inhibited by Reg1Glc7 protein phosphatase and perhaps by a second pathway not involving Reg1.

The roles of Snf1 and Reg1Glc7 in mediating chromatin binding by Adr1 are unknown. One possibility is that Snf1 phosphorylates and activates Adr1, and Reg1Glc7 dephosphorylates and inactivates Adr1. Alternatively, targets of Snf1 and Reg1Glc7 may include proteins that interact with Adr1 and affect its binding to chromatin, or they may include chromatin itself.

Snf1 regulates gene expression in derepressing growth conditions in multiple ways. These include inactivation of the repressor Mig1 (49), activation of the transcription factors Sip4 and Cat8 (7, 50–52), acting through the holoenzyme (53), and modification of chromatin (54). Snf1 activates transcription of INO1 by phosphorylating Ser-10 of histone H3. S10 phosphorylation is necessary for subsequent acetylation of Lys-14 of the same histone (54).

These observations suggest a mechanism for Snf1-dependent chromatin binding by Adr1. The regulatory region of the ADH2 promoter is nucleosome-free, but the proximal Adr1 binding site is immediately adjacent to the upstream border of the TATA-box-containing −1 nucleosome (24, 25). After Adr1-dependent remodeling of the −1 nucleosome is displaced toward the promoter (26) away from UAS1. We hypothesize that the proximity of the −1 nucleosome to UAS1 inhibits Adr1 binding in repressed growth conditions. After glucose depletion, we imagine that phosphorylation of S10 on histone H3 by Snf1 at the ADH2 promoter, and subsequent acetylation of the −1 nucleosome, “loosens” the nucleosome on the DNA and enhances Adr1 binding, leading to nucleosome movement and subsequent PIC formation. Reg1-Glc7 might affect the phosphorylation of histone H3 Ser-10 indirectly, by regulating the activity of Snf1 (4). Alternatively, Reg1-Glc7 might dephosphorylate histone H3 S10-phosphate. The putative Reg1-Glc7-independent pathway of ADH2 repression could act in the same pathway to affect phosphorylation of S10 on histone H3.

The hypothesis that Snf1 acts on Adr1-dependent promoters,
rather than on Adr1 itself, is consistent with several observations. Adr1 isolated from repressed cells is competent to bind DNA in vitro, and its apparent affinity for DNA is not increased after derepression (32). These observations suggest that Adr1 itself may not be in an inactive form in repressed conditions. Rather, the chromatin form of the ADH2 promoter may preclude efficient binding in repressed conditions. Increased levels of Adr1 can partially overcome glucose repression and the requirement for Snf1 (36, 55), suggesting that Adr1 acts downstream of Snf1. In both of these situations constitutive activation is incomplete; removal of glucose enhances expression further. Thus, Snf1-dependent modification required for complete derepression has not been bypassed by increasing the level of the activator. Instead, a low level of activation occurs despite the absence of the modification. This explanation assumes that Adr1 has low affinity for its binding site in vivo in repressed conditions. By increasing the level of Adr1 in glucose-grown cells, mass action would partially compensate for its reduced affinity. Increasing the affinity of Adr1 for its DNA binding site can partially overcome glucose repression, as shown by the constitutive activity of mini3Adr1 in Table III. This explanation is also consistent with the observation that Adr1 is limiting for activation in repressed cells but is present at a saturating level in derepressed cells (33, 36).

The studies presented here show that Adr1 is not bound to chromatin in glucose-repressed conditions. This presents an apparent conundrum for understanding the mechanism of ac- chromatin in glucose-repressed conditions. This presents an apparent conundrum for understanding the mechanism of ac-

Chromatin Binding and in Vitro Transcription by Adr1

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Snf1 Protein Kinase Regulates Adr1 Binding to Chromatin but Not Transcription Activation

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