A Poised Initiation Complex Is Activated by SNF1*\textsuperscript{a}\textsuperscript{b}\textsuperscript{c}\textsuperscript{d}

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Snf1, the yeast AMP kinase homolog, is essential for derepression of glucose-repressed genes that are activated by Adr1. Although required for Adr1 DNA binding, the precise role of Snf1 is unknown. Deletion of histone deacetylase genes allowed constitutive promoter binding of Adr1 and Cat8, another activator of glucose-repressed genes. In repressed conditions, at the Adr1- and Cat8-dependent ADH2 promoter, partial chromatin remodeling had occurred, and the activators recruited a partial preinitiation complex that included RNA polymerase II. Transcription did not occur, however, unless Snf1 was activated, suggesting a Snf1-dependent event that occurs after RNA polymerase II recruitment. Glucose regulation persisted because shifting to low glucose increased expression. Glucose repression could be completely relieved by combining the three elements of 1) chromatin perturbation by mutation of histone deacetylases, 2) activation of Snf1, and 3) the addition of an Adr1 mutant that by itself confers only weak constitutive activity.

The general model for eukaryotic transcription activation is that a preinitiation complex forms by ordered recruitment of specific and general transcription factors to a promoter (see reviews in Refs. 1–3). Early in activation, promoter-specific factors bind, possibly with the aid of general chromatin remodeling factors (4, 5). Bound activators recruit coactivators including histone modifiers and chromatin remodeling complexes such as SAGA\textsuperscript{2} or SWI/SNF. The resulting alterations to chromatin and interactions between bound activator, Mediator complex, and general transcription factors ultimately leads to the recruitment of TBP and RNA Pol II (5–7).

Although the model implies transcription when all components of the preinitiation complex are in place, several cases are known in which Pol II is recruited to a promoter but does not proceed through the open reading frame without an additional signal (8–10). The classic example is the Drosophila hsp70 heat shock gene. In the absence of an activating heat shock and with very little bound heat shock factor, Pol II binds the promoter and initiates transcription, only to pause ~25 nucleotides from the initiation site. Within seconds after heat shock, additional heat shock factor binds, elongation proceeds through the open reading frame (11), and more Pol II is recruited (10). This mechanism allows for a rapid transcriptional response to a potentially lethal environmental challenge.

Reacting to a change in carbon source does not have the urgency of responding to heat shock, and activation of the glucose-repressed genes does not involve a paused polymerase (see review of glucose repression in Ref. 12). We have, however, detected a polymerase complex bound to a glucose-repressed promoter in a strain deleted for the histone deacetylase (HDAC) genes, HDA1 and RPD3. In HDAC mutants, the Adr1 and Cat8 activators, which normally bind to glucose-repressed promoters only in low glucose conditions, bound constitutively. SNF1, which encodes the yeast AMP kinase homolog that is regulated by environmental stresses, was required for the constitutive activator binding as it is for binding in glucose-derepressing conditions (13–15). At the Adr1- and Cat8-regulated ADH2 promoter, a complex was assembled that contained Pol II and components of SAGA, SWI/SNF, TFIIB, and Mediator. Despite the presence of Pol II, transcription was very low. We used the opportunity of a Pol II complex that appeared to be poised for transcription at a glucose-regulated promoter to determine that there are two subsequent steps in the relief of glucose repression, at least one of which can be triggered by activation of Snf1.

**EXPERIMENTAL PROCEDURES**

**Strains and Primers**—Strains are in supplemental Table 1. Yeast strains were grown as described in Sherman (16). Repressing medium contained 5% glucose; derepressing medium contained 0.05% glucose. Epitope tagging, gene deletion, and marker swapping were according to Knop et al., Cross, and Guldener et al. (17–19) respectively, with the exception of SNF1, which was deleted with EcoRI-cut pST70 (20). A multicopy SPT15 (TBP) plasmid was a gift from Steve Hahn’s laboratory.

**ChIP, β-Galactosidase Activity Assay, and Real-time Quantitative PCR**—Chromatin immunoprecipitation and gene-specific PCR with gel electrophoresis were performed as described (21). Antibodies were from Santa Cruz Biotechnology Inc. (9E10 anti-Myc and F7 anti-HA) and Abcam (8WG16 anti-Pol II and Ab5131 anti-CTD phosphoserine 5). For expression analysis, RNA was isolated by hot phenol extraction (22) and converted to cDNA with a SuperScript III kit (Invitrogen).
according to the manufacturer’s directions. ChIP and cDNA were quantified by real-time quantitative PCR (qPCR) with an MJResearch Chromo4 system, using ABI or Quantace SYBR Master Mix, according to the manufacturers’ instructions. Primer sequences are available on request. ChIP data were analyzed using the method of Steger et al. (23) or Bryant and Ptashne (6). β-Galactosidase assays were performed as described in Guarente (24).

*Immunoprecipitations and Western Blots—* Anti-HA and anti-Myc antibodies were from Santa Cruz Biotechnology. Anti-Adr1 was from Dombek et al. (25). Immunoprecipitations were carried out as in Strahl-Bolsinger et al. (26), without DNase I treatment and using 2 μg of monoclonal anti-HA (F-7) or 6 μg of monoclonal anti-Myc (9E10) per mg of lysate. The method of Kushnirov (27) was used for non-immunoprecipitated Western blot samples. Western blots were performed with the Odyssey system (Licor), using 1:500–1:1000 diluted polyclonal anti-HA (Y-11) or monoclonal anti-Myc (9E10) as primary antibody.

**Supercoiling Assay**—The −640 to +135 region of the ADH2 gene was cloned into the multiple cloning site of pALTl, a modified version of pALT (28), resulting in pLLTY1. pLLTY1 was digested with EcoRI, and the resulting 1:300–1:500 and used in qPCR reactions to quantify the presence of a specific amplicon. The protection value set for each amplicon corresponds to the -fold enrichment of that amplicon in the mononucleosomal DNA when compared with the undigested sample and normalized to CEN3 values. qPCR primers were designed to cover the promoter of ADH2 and FBP1 with amplicons averaging 100 bp in size (sequences available upon request).

**RESULTS**

*Mutations in HDACs Cause Activator Binding in Non-activating Conditions*—In glucose-starved cells, Adr1 and Cat8 contribute to the activation of dozens of glucose-repressed genes (21, 31). Neither factor is detected at target promoters in repressed cells by ChIP, but binding is detected after derepression in low glucose (32). In strains deleted for HDA1 and RPD3, Adr1 binds to the ADH2 promoter, even in repressing conditions, without activating transcription (see below and see Ref. 33). We extended this analysis and found that in the HDAC mutants, both Adr1 and Cat8 bound constitutively to the promoters of several Adr1- and Cat8-regulated genes, specifically ACS1, ADH2, ADY2, ATO3, FBP1, JEN1, ICL1, MDH2, MLS1, and PUT4 (Fig. 1 and supplemental Fig. 1).

**Increased Activator Levels in HDAC Mutants Do Not Account for Binding**—For both Adr1 and Cat8, binding under repressed conditions was unexpected. CAT8 expression is dependent on Snf1 (14, 34), which is inactive in vitro when isolated from glucose-grown cells (35). Expression of ADR1 is not SNF1-dependent (36), but Adr1 binding during derepression requires SNF1 (13). HDAC mutations might increase Adr1 and Cat8 protein levels, resulting in binding by simple mass action, so we examined Adr1 and Cat8 levels by Western blot.

Ard1 and Cat8 were present in glucose-grown cells, although as expected from previous results (34, 37, 38), their levels increased dramatically upon derepression (Fig. 2, note that Cat8 samples were concentrated by immunoprecipitation before blotting). Levels of both factors were elevated in ∆hda1∆rpm3 strains (Fig. 2, A and B). Previous work showed that a multicopy ADR1 strain overproduces Adr1 but does not show binding in repressed conditions (13). Fig. 2C shows that although Adr1 in HDAC mutants was elevated, by comparison with the multicopy strain, these levels alone were not sufficient for binding.

*Snf1 Is Required for Activator Binding in HDAC Mutants*—The ∆hda1∆rpm3 mutants appeared to overcome the regula-
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FIGURE 2. Increased Adr1 and Cat8 levels do not account for binding in \( \Delta \text{hda1} \Delta \text{rpd3} \) strains. A, Adr1. 200 \( \mu \)g of lysate from repressed (R) or 6-h derepressed (DR) cells was analyzed by Western blot with monoclonal anti-Myc antiserum. Lane 1, wild type (CTYTY18); lane 2, \( \Delta \text{hda1} \Delta \text{rpd3} \) (CTYTY46); lane 3, \( \Delta \text{hda1} \Delta \text{rpd3} \Delta \text{sfn1} \) (CTYTY51). B, Cat8. 2 mg (lanes marked a) or 1 mg (lanes marked b, lane 4, and 6-h derepressed samples) of lysate was concentrated by immunoprecipitation with monoclonal anti-HA antiserum. Lanes 1, wild type (NKY25b); lane 2, \( \Delta \text{hda1} \Delta \text{rpd3} \) (CTYTY69); lane 3, \( \Delta \text{sfn1} \) (CTYTY77); lane 4, \( \Delta \text{hda1} \Delta \text{rpd3} \Delta \text{sfn1} \) (CTYTY73). R and DR are defined as in panel A. C, Adr1. 100 \( \mu \)g (lanes marked a) or 50 \( \mu \)g of lysate (lanes marked b) from repressed cells analyzed by Western blot with monoclonal anti-Adr1 antiserum. Strains used were \( \Delta \text{hdac} \) (CTYTY44), and multicopy ADR1 (JSY24).

**FIGURE 3.** Pol II and coactivators are recruited to \( \Delta \text{hda1} \Delta \text{rpd3} \) mutants. Binding to the ADH2 promoter was quantitated by qPCR of DNA isolated by ChIP as described under "Experimental Procedures." R, repressed; DR, 4-h derepression. A, binding of Pol II and coactivators. Strains were \( \Delta \text{hda1} \Delta \text{rpd3} \) (RBY75, RBY76, RBY77, RBY88, RBY89, RBY90, RBY91, RBY103, RBY104); \( \Delta \text{hda1} \Delta \text{rpd3} \Delta \text{adr1} \) (ECY2); \( \Delta \text{hda1} \Delta \text{rpd3} \Delta \text{sfn1} \) (RBY82) with tagged coactivators as indicated and in supplemental Table 1. For wild type, each strain was compared with its isogenic \( \Delta \text{hda1} \Delta \text{rpd3} \) parent. ChIP for TBP was from strains carrying an N-terminally Myc-tagged SPT15 on a multicopy plasmid. Data are expressed as the ratio of ChIP/input normalized to a non-expressed telomeric region. The highest value of each set is 100%. The percentage of total immunoprecipitated, normalized to background, B, phosphorylation state of the Pol II CTD. Strains were wild type (CTYTY18) and \( \Delta \text{hda1} \Delta \text{rpd3} \) (CTYTY46). Data are presented as described for panel A and are the average of three biological replicates. \( p = 0.25 \) for hypophosphorylated Pol II at ADH2 in repressed and derepressed \( \Delta \text{hda1} \).
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structure. The supercoiling analysis relies on the fact that the amount of negative supercoiling on an isolated DNA plasmid is proportional to the number of nucleosomes assembled on the DNA in vivo (43). Thus by determining the change in the distribution of topoisomers between samples, one can determine the change in nucleosome density. The -640 to +135 region (relative to the ATG initiation codon) of the ADH2 gene was cloned into a multicopy TRP1/ARS1 yeast plasmid (28). In this plasmid, the cloned promoter, TRP1 and ARS1, can be released by digestion with EcoRI, ligated, and used to transform yeast to Trp prototrophy. The 2.5-kbp episme has the same chromatin architecture as the chromosomal ADH2 promoter, and Adr1-dependent loss of nucleosome density is observed in derepressing conditions.4 When the topoisomer distribution was compared in the wild-type strain between repressed, 2.5-h derepressed, or 5-h derepressed, a downward shift representing loss of one nucleosome occurred between 2.5 and 5 h of derepression (Fig. 5, A and B). This shift in the distribution of topoisomers was not observed when DNA from a Δsnf1 mutant was examined after growth in repressing and derepressing conditions (Fig. 5A, lanes 7 and 8). However, using a Δhda1Δrpd3 strain, the topoisomer distribution seen in the repressed and 2.5-h derepressed samples was similar to the distribution seen at 5 h of derepression in the wild-type strain, indicating that the nucleosome density in the Δhda1Δrpd3 strain in high glucose resembles the wild-type density in low glucose (Fig. 5A, lanes 4 and 5, and B). Upon derepression, there is a greater loss of nucleosomes in the Δhda1Δrpd3 strain (Fig. 5A, lane 6, and B).

The second technique used to detect changes in the chromatin structure was a NuSA. NuSA quantifies the micrococcal nuclease sensitivity of a DNA sequence in vivo and was used to detect nucleosome positioning and density at the ADH2 promoter region. The monosomal DNA resulting from micrococcal nuclease digestion was analyzed using an array of tiled amplicons spanning the ADH2 promoter region and qPCR. The NuSA for wild-type repressed cells showed the three previously described nucleosomes: N-2 at the 5′ end of the nucleosome free region, N-1 covering the TATA box, and N+1 covering the translational start site (42) (Fig. 5C). When wild-type cells were shifted to derepressing conditions, two changes in the chromatin were seen. An overall reduction of nucleosome density was represented by a decrease in the amplitude of the nucleosome protection peaks and a small but reproducible 3′ shift in the position of N-1 (Fig. 5C, inset). Nucleosome density in repressed Δhda1Δrpd3 mutants was comparable with derepressed wild type; however, the small shift in N-1 position was not seen (Fig. 5C). This agrees with data from Verdine et al. (33), showing increased acetylation and micrococcal nuclease sensitivity of the ADH2 promoter in the HDAC mutants. The FBP1 promoter was also analyzed by NuSA (data not shown), and the same trend was seen. There was an overall decrease in nucleosome density in derepressed chromatin when compared with repressed chromatin, and the nucleosome density in the repressed Δhda1Δrpd3 mutant more closely resembled the wild-type derepressed than the wild-type repressed samples.

increase transcription from an ADH2-lacZ reporter in a Δhda1Δrpd3 strain (supplemental Fig. 2).

To evaluate the state of the polymerase poised at the ADH2 gene, we did ChIP for phosphorylation of serine 5 of the CTD, a modification suggestive of initiation (40). The paused polymerase at the Drosophila hsp70, hsp26, GAP, TUB, and Actin5C genes is Ser-5-P-modified (10). In repressed Δhda1Δrpd3 cells, Ser-5-P was detected at the ADH2 promoter at 17% of derepressed levels (Fig. 3B). Hypophosphorylated Pol II, detected with an antibody against unphosphorylated CTD, was present at 42% of derepressed levels, comparable with the total Pol II seen in Fig. 3A. Since different antibodies have different precipitation efficiencies, we could not compare ChIP values directly, but the poised polymerase appeared to be a mixture in which only a fraction was in the Ser-5-P state, measured relative to derepressed levels. By the same criterion, a greater fraction was hypophosphorylated. As a control, CTD Ser-5-P was examined in repressed and derepressed wild-type cells, at the 5′ end of the housekeeping gene ACT1. Levels decreased in low glucose, consistent with reduced transcription during slower cell growth (Fig. 3B).

ADH2 Promoter Chromatin Structure Is Altered in Repressed HDAC Mutants—Transcription of Adr1-dependent genes upon activation in low glucose is associated with an Adr1-dependent chromatin remodeling event at the promoter (41, 42). We used two techniques, supercoiling analysis and NuSA, to determine whether activator binding without low glucose signaling in a Δhda1Δrpd3 strain affected the ADH2 promoter chromatin

4 G. L. Law, unpublished.
Snf1 Activation and a Non-phosphorylatable Adr1 Contribute to Activation—The repressed Δhda1Δrpd3Δ strains presented a unique opportunity for investigating late stage steps in activation of glucose-repressed genes. RNA Pol II and associated transcription factors appeared to be poised at ADH2 and other Adr1- and Cat8-dependent promoters. Some chromatin remodeling had occurred, and possibly some initiation, but glucose repression still persisted.

We hypothesized that one or more final steps, perhaps involving the kinase activity of Snf1, were required for full derepression. Snf1 is kept inactive by the Reg1.Glc7 phosphatase complex (44, 45), so Reg1 was knocked out in Δhda1Δrpd3 and isogenic HDA1 RPD3 strains, and RNA was measured by qPCR. As seen previously (25, 46, 47), in a Δreg1 strain, the Snf1-dependent SUC2 gene was almost fully activated on glucose (80–115% of wild-type derepressed), and ADH2 was expressed to −7% of wild-type derepressed levels (Fig. 6A and data not shown). Low glucose increased ADH2 expression nearly 5-fold, to 33% of wild-type derepressed levels. The generation time of the Δreg1 strain was more than double that of wild type, so slow growth may be responsible for low ADH2 expression. Adding the Δreg1 deletion to an Δhda1Δrpd3 strain showed a clear synergistic effect. Expression of Adr1- and Cat8-dependent genes was as high or higher than a derepressed Δreg1Δhda1Δrpd3 strain. Glucose repression still prevailed for all genes tested except SUC2 as further activation was seen after removing glucose (Fig. 6A and data not shown for the Adr1- or Cat8-bound ADY2, IC12, FBP1, and MLS1 genes).

Another activation event that might be absent in the Δhda1Δrpd3 mutants involves Adr1. Although the precise mechanism is unknown, mutations in the region of the phosphorylated serine 230 in Adr1 relieve glucose repression of ADH2, leading to up to 10% of fully derepressed levels (48, 49). We confirmed this by qPCR on transcripts from an Δadr1 strain with the ADR1 S230A allele (Adr1c) on a CEN plasmid. Comparable with earlier results (25), the ADR1c allele caused constitutive expression of ADH2 but to 2% or less than wild-type derepressed levels (Fig. 6A). It had a hyperactivating effect in derepressing conditions, even for FBP1 and MLS1 (data not shown), which are not strongly Adr1-dependent, although Adr1 can be detected at their promoters (32). Adr1c had a greater than additive effect when combined with the Δhda1Δrpd3 mutations, and hyperactivation was seen when glucose was depleted (Fig. 6B).

Dombek et al. (25) showed that combining the ADR1c allele and Δreg1 caused constitutive ADH2 expression to 20% of wild-type derepressed levels, indicating that Reg1 and Adr1c act in separate pathways. Lack of full expression suggested other influences on expression. We added the dominant (48, 49) ADR1c allele on a plasmid to the Δreg1 strains used in Fig. 6A and found that it had the expected synergistic effect at ACS1, ADH2, and FBP1 (Fig. 6B and data not shown). At ADH2 and ACS1, expression in glucose was 300% of wild-type derepressed levels, yet glucose repression was still operational because shifting cells to low glucose gained further expression.

5 J. J. Infante, unpublished.
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**DISCUSSION**

Chromatin Perturbation Allows Activator and Preinitiation Complex Binding at ADH2—Consistent with current models of coactivator factor bound its target promoter ADH2, it recruited a partial preinitiation complex that included Pol II, even in nonactivating conditions. Some CTD Ser-5-P could be detected, and although this is not definitive evidence of initiation, it suggested that at least some of the Pol II was transcription-competent. Binding of coactivators and Pol II after a nonspecific chromatin perturbation was consistent with recent data on the phosphate-repressible PHO5 promoter, where initial chromatin remodeling by SWI/SNF and SAGA, and later remodeling by the histone chaperone Asf1, are key events in activator binding and subsequent transcription complex assembly (4). Zhang and Reese (50) showed that disrupting a positioned nucleosome over the TATA box of the DNA damage-inducible RNR3 led to Pol II recruitment in repressed conditions, although in this case, significant transcription was achieved. Some chromatin remodeling of the ADH2 promoter had occurred in the HDAC mutant, but the TATA-containing nucleosome had not been repositioned as it is in derepressed cells.

Recruitment of coactivators and Pol II demonstrates that the Adr1 activation domain(s) are functionally accessible in repressing conditions unlike the activation domains of some inducible transcription factors (e.g. peroxisome proliferator-activated receptors). As in derepressing conditions, formation of the partial initiation complex in the HDAC mutant required activator, and activator binding required SNF1. Nevertheless, the presence of this complex was insufficient for full transcription, and the promoter was still subject to glucose repression.

**Snf1 Has Activities in Glucose-grown Cells**—The SNF1 requirement for binding at glucose-repressed genes in repressing conditions was surprising because Snf1 is considered to be inactive at these promoters in these conditions. Although Snf1 purified from glucose-grown cells does not show *in vitro* kinase activity against a peptide substrate (35), SNF1 is required for full INO1 expression when it is induced by low inositol, even in the presence of glucose (51–53). In repressed cells, Snf1 coimmunoprecipitates with its substrate Mig1 (54), indicating some interaction with target proteins, even when it may not be catalytically fully active. We have seen that Δsnf1 increases the proportion of Adr1 that is phosphorylated on Ser-230 in both glucose-repressing and low glucose-derepressing conditions, although the effect on DNA binding, coactivator interaction, and transcription activation is still under investigation. Recently, Hong and Carlson (55) have shown that the kinase activity of Snf1 is activated in glucose-grown cells that are subjected to high pH or high NaCl. Constitutive, Snf1-dependent DNA binding of Adr1 and Cat8 when promoter chromatin structure is altered might be because the Δhdr1Δrpd3 mutations introduce a particular cell stress that partially activates Snf1.

**Snf1 Acts at an Activation Step after RNA Pol II Binding**—We hypothesize that a Snf1-dependent step occurs downstream of
Pol II recruitment since adding a REG1 deletion to the histone deacetylase deletion strains had a greater than additive effect on transcription than either of the mutations alone. We believe that Snf1 activation, rather than a pleiotropic effect of Δreg1, is responsible. REG1 deletion is commonly used to deregulate Snf1. Snf1 is activated through phosphorylation of its serine 210 (35, 56–59) and inactivated through dephosphorylation by a PP1 type 1 protein phosphatase composed of Glc7 and the regulatory subunit Reg1 (35, 45, 56). As a PP1 phosphatase, Glc7 has many substrates (60) and therefore pleiotropic effects. Reg1, however, as the subunit that targets Glc7 to Snf1 (44, 61), has many substrates (60) and therefore pleiotropic effects. As confirmation to the role for Snf1 is reverse recruitment, transporting genes to the nuclear periphery during derepression (68). Further steps that are triggered by low glucose and may or may not involve Snf1 occur beyond the late Snf1-dependent step since a shift to low glucose induced further gene expression. The Adr1 activator itself is implicated since an S230A mutant allowed full expression in the Δreg1Δhda1Δrpd3 strain.

Our model (Fig. 7) is that, in repressed Δhda1Δrpd3 cells, Snf1 is sufficient to allow binding of Adr1 and Cat8. Deletion of REG1 either increases the amount of active Snf1 or stimulates a different activity of Snf1 that leads to activation-competent Adr1 and Cat8. For example, Snf1 may be required for a post-binding phosphorylation of Cat8 that leads to activation of transcription. Unphosphorylated Cat8 appears to be competent to bind because Heisinger et al. (69) found that bacterially produced Cat8, which is not expected to receive its normal modifications, showed in vitro DNA binding. Phosphorylation is important to the activator function of Cat8 because a

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