Factors Affecting Saccharomyces cerevisiae ADH2 Chromatin Remodeling and Transcription*

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The chromatin structure of the Saccharomyces cerevisiae ADH2 gene is modified during the switch from repressing (high glucose) to derepressing (low glucose) conditions of growth. Loss of protection toward micrococcal nuclease cleavage for the nucleosomes covering the TATA box and the RNA initiation sites (−1 and +1, respectively) is the major modification taking place and is strictly dependent on the presence of the transcriptional activator ADR1.

To identify separate functions involved in the transition from a repressed to a transcribing promoter, we have analyzed the ADH2 chromatin organization in various genetic backgrounds. Deletion of the CCR4 gene coding for a general transcription factor impaired ADH2 expression without affecting chromatin remodeling. Growing yeast at 37 °C also resulted in chromatin remodeling at the ADH2 locus even under glucose repressing conditions. However, although this temperature-induced remodeling was dependent on the ADR1 protein, no ADH2 mRNA was observed. In addition, inactivating RNA polymerase II (and therefore, elongation) was found to have no effect on the ability to reconfigure nucleosomes. Taken together, these data indicate that chromatin remodeling by itself is insufficient to induce transcription at the ADH2 promoter.

Transcriptional regulation has been extensively studied both by genetic and biochemical approaches. The overall complexity of the eukaryotic genome and the large number of genes to be regulated are such that repression and chromosome compaction are necessarily the dominant feature. According to this concept, the wrapping of DNA around nucleosomes is considered the easiest way to keep the majority of the genome silent. Activation would then be achieved by different combinations of positive factors.

During the last 10 years, a more complex picture relating chromatin to gene activation has emerged (1–4). Extensive work by Grunstein and co-workers (Refs. 5 and 6 and references therein) has pointed out that each specific histone is involved in the activation or repression of defined genes and that different histone domains have different regulatory functions.

As a consequence, nucleosomes would hold more important functions than just folding and wrapping the huge mass of eukaryotic DNA. A direct involvement of specific core particles in gene activation is suggested by the finding that multiple factors acting on a promoter interact successfully with each other due to the presence of a precisely positioned nucleosome (7, 8). In this context, the in vivo study of the exact location of nucleosomes (9–11) as well as the in vitro analysis of the rules governing their positioning (12–16) become relevant for the understanding of regulatory mechanisms.

Given that transcriptional control cannot be studied without considering chromatin as the real template for RNA polymerase II, especially because in many cases key promoter elements are occupied by nucleosomes (17–19), many recent studies have concentrated on identifying the protein complexes capable of unfolding or modifying chromatin structure (20, 21). These protein complexes have been found to act on specific genes and appear to be conserved among eukaryotic organisms (22, 23), suggesting that overcoming the nucleosomal barrier is indeed an important step in transcriptional activation.

Nucleosomes have also been shown to possess special features that facilitate their recognition and aid in allowing access of the transcription initiation complex. Acetylation of specific histone residues have been implicated in targeted nucleosome disruption (24–26) during activation processes, whereas the reverse reaction, deacetylation, appears to be required to ensure repression (27, 28).

We have chosen as a model system the promoter of the Saccharomyces cerevisiae ADH2 gene coding for the enzyme alcohol dehydrogenase II (29) to answer two questions concerning the role of chromatin in gene activation. Is chromatin simply an obstacle for promoter recognition that once removed automatically leads to transcription? Given this context, what is the function of transcriptional activators? Previously, we have shown that a defined nucleosomal organization characterizes the ADH2 promoter in repressing (high glucose) conditions. Upon changing the medium to derepressing (low glucose) conditions, a modification occurs in the protection pattern of two nucleosomes that cover the basal transcription elements (TATA box and RNA initiation sites) (30). This chromatin remodeling requires the product of the ADR1 gene (30), coding for a zinc finger protein known to activate ADH2 and genes involved in peroxisomal function (31, 32).

Genetic evidence indicates that at least two regulatory pathways are required for full ADH2 expression. Besides the transcriptional activator ADR1, which acts through the upstream activation sequence 1 region (33), a second pathway consisting of the activator CCR4 and the negative effectors SPT10 and

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SPT6 do not require specific upstream activation sequences (34). Both activators are likely to interact with additional cofactors, ADR1 contacting ADA2 and GCN5, components of a histone acetylase complex (35), whereas CCR4 is part of a multisubunit transcriptional regulatory complex (34).

Here we investigate the role of different elements of the ADH2 system (the positive factors CCR4 and ADR1, RNA polymerase II) by analysis of specific mutants to determine whether nucleosomal reconfiguration represents a necessary and sufficient event for promoter activation. We find that CCR4 is not required for altering chromatin structure but rather appears to control the transcription rate. ADR1, in contrast, is found to play a role in nucleosomal disruption even when this is induced by a signal (temperature increase) that does not lead to ADH2 mRNA accumulation. We also report that the catalytic activity of RNA polymerase II is not relevant for the maintenance of the chromatin alteration. As a whole, these results indicate that the modification of nucleosome structure at the ADH2 promoter is an important step in the activation process, although not sufficient for transcription.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—Yeast strains are listed in Table I. Strain BY260 is described in Ref. 36. Strains HD93-15D and HD93-15DbB, kindly provided by G. Pereira and C. P. Hollenberg, are described in Ref. 37.

Yeast cells were grown in 1% yeast extract, 2% peptone, 3% glucose as rich medium. ADH2 derepression was obtained as follows. Cells grown overnight in 200 ml of medium containing 3% glucose (to 0.5 optical density units/sample) were pelleted and resuspended in 5–8 ml of Zymolyase buffer (39). Cells (25–40 optical density units/sample) were pelleted and resuspended in the same volume of fresh medium containing 0.05% glucose. At different times, samples were collected, and both chromatin and RNA were analyzed. Temperature shifts were performed as follows. Cells grown overnight at 24 or 30 °C were pelleted and resuspended in the same volume of fresh medium equilibrated at 37 °C.

Plasmid—The 2,250-base pair BamHI fragment shown in Fig. 1 and described in Ref. 30 was inserted in the HindIII site of PUC18SM (38) to obtain plasmid pFA. This plasmid DNA was used to prepare probes for the indirect end-labeling analysis and as deproteinized material for control reactions with micrococcal nuclease (MN).1 For the ADH2 sequence, see Ref. 29.

Enzymes—All nucleases were purchased from Boehringer Mannheim, and Zymolyase 100T was purchased from Seikagaku Corp.

Chromatin Analysis—The method is based on MN treatment of nystatin-permeabilized spheroplasts (39). Cells (25–40 optical density units/sample) were pelleted and resuspended in 5–8 ml of Zymolyase buffer (1 M sorbitol, 50 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol) in the presence of 0.05 mg of Zymolyase 100T/optical density unit. Incubation was carried out for 30 min at 30 °C. For the cells grown at 37 °C, incubation was at 37 °C. The pelleted spheroplasts were resuspended in nystatin buffer (50 mM NaCl, 1.5 mM CaCl2, 20 mM Tris-HCl, pH 8.0, 1 M sorbitol, 100 μg of nystatin/ml) and divided into 0.25 ml aliquots. MN (0.5–10 units) was added to each aliquot of permeabilized spheroplasts, and incubation was performed at 37 °C for 15 min. The reaction was stopped with 1% SDS and 5 mM EDTA (final concentrations). Proteinase K (40 μg/sample) was added, and the samples were kept at 56 °C for 2 h. The DNA was then purified with phenol-chloroform extractions (three times) and ethanol precipitation. In vitro treatment of samples with MN was as follows. Chromosomal or plasmid DNA was reacted in 0.1 ml of MN digestion buffer (50 mM NaCl, 1.5 mM CaCl2, 20 mM Tris-HCl, pH 8.0) with 0.25–2 units of MN for 3 min at 37 °C. The reaction was stopped with 1% SDS and 5 mM EDTA (final concentrations) and purified by phenol-chloroform extraction and ethanol precipitation.

Indirect end-labeling analysis (40) was performed as follows. After secondary digestion with BamHI and/or HindIII, the samples were electrophoresed in 1.5% agarose-Tris borate-EDTA (TBE) gels (1.75 V/cm), transferred to nitrocellulose paper (Schleicher & Schuell), and hybridized with probes 5′ or 3′, respectively, by standard procedures.

RNA Analysis—For each experiment, aliquots containing an equal number of cells (0.5–1 × 106) were pelleted, and total RNA was prepared as described in Ref. 41. After spectrophotometric determination of the amount of RNA present in each aliquot, 10–20 μg of RNA were loaded onto 1.2% agarose-MOPS gels containing formaldehyde as a denaturing agent and ethidium bromide as an intercalating dye.

Northern blot analysis was performed by standard procedures with Hybond N+ nylon paper (Amersham). For hybridization, a 5′-end-labeled oligonucleotide specific for the ADH2 gene was used. Map positions and sequence are as follows. From +710 to +864, 5′-GTTGGTGTACCTTAAAGCTGCCGTAAC-3′.

RESULTS

The structure of the ADH2 promoter and the positioning of the three relevant nucleosomes (−2, −1, and +1, numbered relative to the RNA initiation sites) are shown in Fig. 1. The borders of the multiple alternative particles have been determined by in vivo high resolution mapping on isolated monomeric DNA (30). The chromatin organization presented in Fig. 1 characterizes the ADH2 promoter in repressing (high glucose, 3%) conditions. Upon changing the medium to derepressing (low glucose, 0.05%) conditions, the two nucleosomes covering the TATA box and the RNA initiation sites (−1 and +1, respectively) underwent a rearrangement, causing loss of protection of the underlying sequences (30). To determine which proteins (e.g. general or specific transcription factors, RNA polymerase II) were involved in this remodeling event, we have analyzed the ADH2 promoter chromatin structure in various genetic backgrounds.

Table I

| Strain | Genotype |
|--------|----------|
| EGY188 | MATa his3 leu2 trp1 ura3 leuAop-LEU2 |
| EGY188-1 | Same as EGY188 except cer4::URA3 |
| BY260 | MATa ura3-52 rpl1-1 |
| HD93-15D | MATa his3 leu2 trp1 ura3 |
| HD93-15DbB | Same as HD93-15D except adr1::HIS3 |
| H34-60 | MATa his4 trp1 ura1 adh1-11 adr1::ADR1-5′-TRP1 |
| R222-2-8B | MATa ade2 trp2 ADH2-4 |

1 The abbreviations used are: MN, micrococcal nuclease; MOPS, 4-morpholinopropanesulfonic acid.

Fig. 1. Schematic map of the ADH2 gene. The positions of the relevant elements are given relative to the ATG (A = position +1). RIS, RNA initiation sites. Probe 5′, BamHI-XbaI fragment, 130 base pairs. Probe 3′, TaqI-HindIII fragment, 102 base pairs. Each group of ovals represents a family of multiple overlapping nucleosomes whose borders (bars above the line) were mapped at high resolution (30).
Nucleosome Modification and Promoter Activation

CCR4 Is Not Required for the Change in Chromatin Structure at the ADH2 Promoter—One of the factors known to be involved in ADH2 transcription is the product of the CCR4 gene coding for a glucose-regulated protein, originally identified as a suppressor of mutations in the SPT6 and SPT10 genes that allow increased transcription at ADH2 (42). Given that SPT6 has been implicated in nucleosome assembly (43), we wanted to analyze the role of CCR4 in chromatin organization. We analyzed ADH2 chromatin structure in two isogenic strains, one of which carried a CCR4 gene disruption. The results are shown in Fig. 2. In repressing conditions (Fig. 2A, G = 3% glucose) in both the wild-type and the ccr4 mutant strains, the area occupied by the nucleosomes −1 and +1 was protected (small filled circles) point out the strong MN cleavage sites found on naked DNA approximately at the level of the TATA box and the ATG consensus sequences; they are covered in the samples from repressed cells). When the cells were grown in derepressing conditions (Fig. 2A, E = 3% ethanol) or shifted to low glucose (Fig. 2B, 0.05%G), loss of protection was observed for both strains, indicating that the two nucleosomes became modified. Two additional nucleosomes, −2 and +2, as previously reported (30), became modified (Fig. 2A, lanes E). The extent and the kinetics of this promoter modification were found to be very similar in both strains (compare, for example, Fig. 2B, lanes 1 h at 0.05%G). By contrast, the amount of steady state mRNA was substantially lower in the ccr4 mutant strain, which was found to protect the nucleosome −1 and +1 was protected (small filled circles). Fig. 2D indicates that ADH2 mRNA accumulation shown in Fig. 2D indicated that ADH2 mRNA appears earlier in the absence of CCR4 (30 min time point), after which it slightly increases, never reaching the abundance observed in the wild-type strain. We conclude from these experiments that CCR4 is required downstream of the chromatin remodeling event to allow efficient transcription.

Induction of Chromatin Remodeling in an RNA Polymerase II Temperature-sensitive Mutant Strain—The experiments reported in Fig. 2 show that the transcription rate is not correlated with the extent of nucleosome reconfiguration. In fact, the structural chromatin change occurs with the same efficiency irrespective of the mRNA amount produced. To further test the role of transcription in the remodeling process, we analyzed the chromatin structure at the ADH2 promoter in an rpb1-1 strain containing an RNA polymerase II temperature-sensitive mutation (36). When the temperature is increased to 37 °C in strains carrying this mutation, a block in the elongation ability of RNA polymerase II immediately occurs (36). This mutation impairs the catalytic activity of the enzyme but does not influence the assembly of the subunits. Fig. 3A shows the results of this analysis. At the permissive temperature (24 °C) and in repressing conditions (3% glucose), the nucleosomes −1 and +1 were found to protect the ADH2 promoter. Surprisingly, the chromatin structure was found to be modified after 1 h of incubation at the nonpermissive temperature (37 °C), even under glucose-repressing conditions (lanes 3% G, 37 °C). If the cells were then washed and incubated in 0.05% glucose-containing medium at the same restrictive temperature, the chromatin remained open, whereas no trace of ADH2 mRNA was observed (see Fig. 3B).

Two main facts emerge from this experiment. (i) Transcriptional elongation is not required to actively maintain the nucleosomes in the modified state; on the other hand, assembly of the pre-initiation complex, which is not impaired in the rpb1-1 strain at 37 °C, may still be responsible for the nucleosomal modification, and (ii) an environmental stimulus (heat), not apparently related to glucose metabolism, is able to induce the chromatin change at the ADH2 promoter.

Nucleosome Rearrangement Can Be Induced in Vivo by Increasing the Temperature. This Effect Is ADR1-dependent but Does Not Lead to ADH2 mRNA Accumulation—To exclude the possibility that the nucleosome modification shown in Fig. 3A (37 °C, 3% G) occurred fortuitously during the inactivation of the RNA polymerase II catalytic subunit, we analyzed the effect of temperature on chromatin remodeling in a wild-type strain containing a functional enzyme and, at the same time, we asked whether this effect requires the major ADH2 activa-
tor protein, ADR1. The results are shown in Fig. 4. Cells grown overnight at 30 °C in repressing conditions (3% glucose) were pelleted and resuspended in fresh medium equilibrated at 30 °C or 37 °C. After incubation at these two temperatures for 1 h without changing the glucose content of the medium, the chromatin was analyzed using the 3′ probe. The two bands indicating loss of nucleosome protection became visible only in the case of the wild-type strain, suggesting that the temperature-induced chromatin remodeling occurs also in a normal strain and requires ADR1. The result was confirmed using the 5′ probe (data not shown).

We then asked whether this temperature-induced effect represents an alternative way to induce ADH2 expression in the wild-type strain, and we found that this is not the case. Fig. 5 shows that no ADH2 mRNA accumulation was observed even after 3 h of incubation at 37 °C, if the cells were kept in 3% glucose. By contrast, when the cells incubated at 37 °C for 1 h in 3% glucose were shifted to 0.05% glucose-containing medium at the same temperature and tested at 1 and 3 h, ADH2 mRNA was detected. The kinetics of mRNA accumulation at this temperature were different from what is normally observed when cells are shifted to 0.05% glucose at 37 °C (30); here, the steady state mRNA level peaked at 1 h and thereafter decreased.

These results indicate that the chromatin structure of the ADH2 promoter is affected by at least two different signals: glucose reduction, which leads to transcriptional activation, and temperature increase, which does not.

In addition, we observe that transcription is not required for chromatin remodeling at this promoter. Under two circumstances, when there is absolutely no ADH2 transcription (shift to 37 °C under glucose growth conditions and derepression in the presence of the defective rpb1–1 allele; see above), nucleosomal remodeling occurs and is maintained.

A Point Mutation in ADR1 Allows Chromatin Remodeling and Partially Constitutive Expression in High Glucose—The results shown in Figs. 4 and 5 imply that, in the presence of high glucose, ADR1 induces the nucleosome modification if the temperature is raised, but its function as a transcriptional activator is still repressed. We analyzed a mutant strain carrying the ADR1–5′ allele, which allows partially constitutive expression of the ADH2 gene because of an Arg to Lys substitution in position 228 in ADR1 (44). Fig. 6 shows the ADH2 chromatin structure in the ADR1–5′ strain grown in 3% glucose. The ADH II activity in this strain is already high in 3% glucose and becomes higher than a corresponding wild type when the glucose is lowered (45). It can be seen from Fig. 6 that the chromatin structure of the promoter was disrupted under...
glucose growth conditions, suggesting that remodeling and transcription can be achieved in high glucose and at the normal temperature as long as the repression exerted on a specific domain of ADR1 is abolished.

**DISCUSSION**

The first event in the transcriptional regulation of any gene is the processing of extracellular and/or intracellular signals, whereas the last phase consists of changes in the rate of transcription. To understand all the processes that happen in between, both *in vitro* and *in vivo* approaches need to be utilized.

We have used a strategy of genetic dissection to analyze one particular moment in the process of transcriptional activation: the chromatin modification occurring at the *S. cerevisiae ADH2* promoter when the cells are shifted from repressing to derepressing conditions of growth. This study has been carried out in different genetic backgrounds with two types of assays: indirect end-labeling analysis of MN-treated spheroplasts to look at specific nucleosomes and Northern analysis to check the extent of mRNA accumulation in the same cells from which spheroplasts are prepared. The rationale of this work was the examination of mutants belonging to different groups of proteins to identify those involved in the chromatin remodeling event.

**Two-step Model for ADH2 Transcriptional Activation**—The results presented in this work outline a model for *ADH2* promoter activation in which at least two separate steps can be identified, as shown in Fig. 7.

In the first step (repression → derepression), the structure of the nucleosomes -2, -1, and +1 located on the relevant promoter elements undergoes a remodeling event characterized by loss of protection of the underlying sequences. For this event to occur, the transcriptional activator ADR1 is required, and the repression exerted on the protein must be relieved. This can be achieved by several means: (i) by lowering the amount of glucose in the medium, (ii) by increasing the temperature from 30 to 37 °C, or (iii) by mutating ADR1 in the 227–239 region (46).
The catalytic activity of RNA polymerase II is not required for ADR1-mediated nucleosomal rearrangement (Fig. 3), whereas it is likely that the binding of the basal transcription factors and/or the assembly of the holoenzyme are involved because of the dependence on ADR1. Recent evidence suggesting an interaction between ADR1 and TFIID supports a role for ADR1 in recruiting the transcriptional machinery (35). The requirement of the transactivation domain of the activator protein Pho4 in the remodeling process occurring at the PHO5 promoter (47) and the fact that a single contact with the RNA polymerase II holoenzyme is sufficient for the remodeling at that promoter (48) is in line with this evidence.

In the second step (derepression → transcription), transcription commences and, in the presence of the positive factors CCR4 and presumably ADR1, leads to full-level mRNA accumulation, whereas the chromatin remains in the open configuration.

The two-step model outlined for the ADH2 promoter has been already established in vivo for other yeast genes like SUC2 (49), PHO5 (50), and GAL1 (51) by using TATA box mutants to affect the transcription level. However, in the case of the ADH2 promoter (this work), the TATA box is integral, and all the other promoter elements have been retained in their normal chromosomal location.

In no case do we observe transcription without nucleosome disruption. On the other hand, the chromatin remodeling by itself is not sufficient to allow maximal level of ADH2 mRNA accumulation. In fact, we find that (i) the heat-induced chromatin remodeling is not followed by ADH2 mRNA synthesis as long as the glucose content of the medium is kept high (Figs. 4 and 5), and (ii) in the absence of CCR4, the ADH2 mRNA level is low even though the extent and the kinetics of nucleosome disruption do not differ from a wild-type isogenic strain (Fig. 2).

Role of ADR1 and CCR4—According to the working model shown in Fig. 7, we predict that when the glucose level becomes sufficiently low ADR1 primes the assembly of the pre-initiation complex, and at the same time, the disruption of the two nucleosomes (–1 and +1) protecting the TATA box and the RNA initiation sites (Fig. 7, first step). The assembly of the pre-initiation complex proceeds via initial recruitment of TFIID and subsequent recruitment of the holoenzyme, as recently proposed (52). The fact that part of the TFIID structure closely resembles the histone octamer structure (53, 54) suggests a speculative model of molecular substitution. In the second step (Fig. 7), transcriptional elongation takes place only if additional negative effectors are removed; part of this repression is exerted again on ADR1 and part on CCR4. The transcriptional activator CCR4 is, in fact, glucose-regulated and has been found to be part of a multisubunit transcriptional regulatory complex (34, 55). More recently, a functional interaction between the CCR4 complex and a cell-cycle-regulated protein kinase has been reported (56). Perhaps CCR4 contacts some component of the holoenzyme and introduces a stabilizing effect. In fact, in its absence, the sudden increase in ADH2 mRNA accumulation that occurs in a wild-type strain after the start of transcription is not observed (Fig. 2, panel D).

The known genetic interaction of SPT6 and CCR4 is also clarified by the data presented herein. Mutations in CCR4 and SPT6 (a component of the CCR4 complex) (55) are the only known mutations that reduce the ability of sp6 defects to allow increased transcription (42, 55, 57). SPT6 has been shown to bind histones and, when mutated, to result in nucleosomal rearrangement (43). The increased transcription resulting from sp6 mutations is therefore consonant with its effects on nucleosomal rearrangement. Once this rearrangement occurs there may be several different pathways for activation, perhaps depending on how the transcriptional initiation complex is stabilized. Alternatively, the chromatin conformation at the ADH2 promoter caused by mutant alleles such as spt6 and ADR1–5’ or by other factors like glucose or high temperature may all not be physically identical.

ADR1 and the Effect of Heat—We have also found that the ADR1 protein is required for the heat-induced chromatin disruption (Fig. 4). This result is intriguing; the same protein appears to be the target of two different extracellular signals. In looking for a common feature to these signals, it may be possible that glucose decrease in the medium is a stress just as temperature increase. Different stress conditions might trigger a general response by creating the same intracellular signal (for a discussion see Ref. 58). We are currently trying to understand whether ADR1 can work as a mediator of stress effects (to be detailed elsewhere).

The two events leading to chromatin remodeling (low glucose and temperature increase) can follow two different pathways, converging on the same molecule (ADR1) but involving different domains of the protein. Alternatively, the pathway is the same; the two extracellular signals are transduced very soon to the same molecule, which leads to the ADR1-dependent nucleosome change, but glucose still controls the transcriptional ability of the activator. Evidence for multiple levels of control of ADR1 have indeed been presented (59). It cannot be excluded, however, that the two chromatin modifications are structurally different. High resolution analysis will help to clarify this point.

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