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Chromatin Remodeling during *Saccharomyces cerevisiae* *ADH2* Gene Activation

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We have analyzed at both low and high resolution the distribution of nucleosomes over the *Saccharomyces cerevisiae ADH2* promoter region in its chromosomal location, both under repressing (high-glucose) conditions and during derepression. Enzymatic treatments (micrococcal nuclease and restriction endonucleases) were used to probe the in vivo chromatin structure during *ADH2* gene activation. Under glucose-repressed conditions, the *ADH2* promoter was bound by a precise array of nucleosomes, the principal ones positioned at the RNA initiation sites (nucleosome +1), at the TATA box (nucleosome −1), and upstream of the ADR1-binding site (UAS1) (nucleosome −2). The UAS1 sequence and the adjacent UAS2 sequence constituted a nucleosome-free region. Nucleosomes −1 and +1 were destabilized soon after depletion of glucose and had become so before the appearance of *ADH2* mRNA. When the transcription rate was high, nucleosomes −2 and +2 also underwent rearrangement. When spheroplasts were prepared from cells grown in minimal medium, detection of this chromatin remodeling required the addition of a small amount of glucose. Cells lacking the ADR1 protein did not display any of these chromatin modifications upon glucose depletion. Since the UAS1 sequence to which Adr1p binds is located immediately upstream of nucleosome −1, Adr1p is presumably required for destabilization of this nucleosome and for aiding the TATA-box accessibility to the transcription machinery.

Many different proteins are involved in the transcriptional regulation of eukaryotic cells. Genes which must be activated only under particular spatial and/or temporal conditions need to be maintained in a repressed configuration until the advent of a specific inducing signal. The strategy adopted in several cases makes use of a group of evolutionarily conserved proteins: the histones. Evidence obtained in the last 10 years has shown that the role of these proteins is not only a structural one: histones are also directly involved in regulation (29, 37, 39, 61, 75).

Nucleosomes have been shown to work as repressors of promoter function both in vitro (35, 45, 76) and in vivo (30, 47, 58). Interestingly, in some instances, they favor transcription initiation by locating relevant sequences on their surface (51, 62, 65). Direct interactions between defined histone residues and regulatory factors have been reported (32).

Thus, knowing the exact positions of nucleosomes with respect to relevant promoter elements is a fundamental piece of information for the comprehension of basic control processes (8, 9, 16, 25, 43, 54, 61).

In the yeast *Saccharomyces cerevisiae*, several gene systems possess a well-defined chromatin organization which undergoes rearrangements at the time of promoter activation, either before or during the transcriptional process (3, 5, 11, 23, 24, 34, 38, 40). The molecular details underlying nucleosome destabilization during transcription are still poorly understood: octamer disruption and/or chemical modifications of individual histones have been hypothesized (1, 67).

Much interest has been focused recently on gene activation mechanisms involving the SWI-SNF protein complex (42, 63). These proteins are considered general activators of transcription not only in *S. cerevisiae* but also in *Drosophila melanogaster*, mouse, and human systems (reviewed in reference 10). It has been proposed that the SWI-SNF complex mediates chromatin remodeling by gene-specific activators in an ATP-driven fashion (41). In the case of the yeast *SUC2* gene, coding for a glucose-repressed invertase, the involvement of this complex in controlling chromatin organization in vivo has been clearly demonstrated (33).

We have undertaken the analysis of the nucleosome organization in the promoter region of the *S. cerevisiae ADH2* gene, coding for the glucose-repressed alcohol dehydrogenase II (79). Along with the dependence of its transcriptional activation on a functional SWI-SNF complex, mainly in the presence of a Ty insertion in the promoter region (13, 41), *ADH2* gene expression requires the general transcription factor CCR4, which has been implicated in the maintenance of chromatin structure (17, 20). In addition, previous studies have indicated an increased DNase I sensitivity of the *ADH2* gene under derepressing conditions (57). We have chosen the *ADH2* gene as a model system to investigate the relationship between chromatin structural organization and promoter function. We present a detailed analysis of nucleosome positioning over the relevant regulatory sequences in the presence of glucose (repressing conditions), and we show that the nucleosomes covering the TATA box and the RNA initiation sites (nucleosome −1 and +1, respectively) undergo a conformational change when the glucose level in the medium is reduced (derepressing conditions). This change is dependent on the presence of Adr1p in the cells.

MATERIALS AND METHODS

Yeast strains and growth conditions. Strain CH335 (**a** *his4 lys2 ura3*) was kindly provided by J. C. Wang; strain HD93-15D (**a** *his3 leu2 ura3 trp1*) and its isogenic strain HD93-15DdB (**a** *adr1*Δ*::HIS3 leu2 ura3 trp1*) were provided by G. Pereira and C. Hollenberg. *ADR1* gene disruption was obtained as follows. Plasmid YRp7-*ADR1*-5c-23A (18) was cut with *Bcl*I at positions +441 and +1074 of the *ADR1* sequence, and this region was replaced with a 1.7-kb DNA fragment

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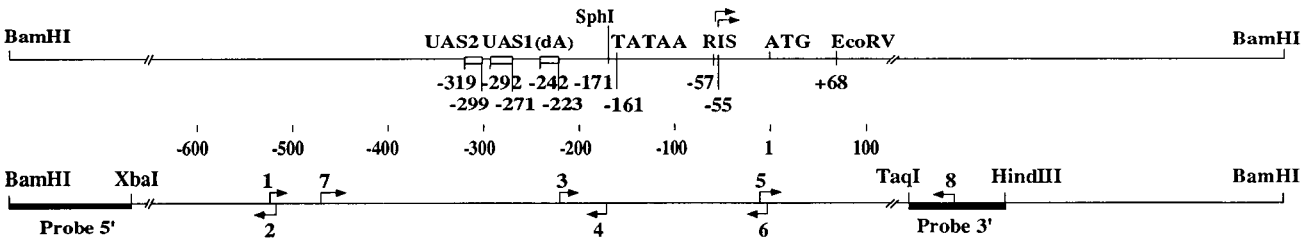

FIG. 1. Schematic map of the *ADH2* gene. The positions of the relevant elements are given relative to the ATG (+1). RIS, RNA initiation sites. The probes used for indirect end labeling and the oligonucleotides used for the high-resolution analysis are shown. Probe 5′, *Bam*HI-*Xba*I fragment, 130 bp; probe 3′, *Taq*I-*Hin*dIII fragment, 102 bp. For each oligonucleotide, map positions and sequences are as follows: 1, from −526 to −508 (5′CTGATGCCAAGAACTCTAA3′); 2, from −519 to −540 (5′GGCATCAGAAAATTTGAGAAAC3′); 3, from −219 to −198 (5′AGAGGAGAGCATAGAAATGGGG3′); 4, from −170 to −191 (5′GGCATGCTA TAGCTTTACCAAAA3′); 5, from −12 to +12 (5′TGCTAATACACAATGTCTATTCCA3′); 6, from +5 to −21 (5′GACATTGTGTATTACGATATAGTTAA3′); 7, from −472 to −448 (5′CTCCGGTTACAGCCTGTGTAACTGA3′); 8, from +710 to +684 (5′GTTGGTAGCCTTAACGACTGCGCTAAC3′).

bearing the *HIS3* gene. From this construct, a 3.4-kb *Pvu*I-*Pvu*II fragment (positions −310 to +1894 of *ADR1*) was isolated and used to disrupt the *ADR1* gene in strain HD93-15D. The resulting strain was designated HD93-15DdB (39a).

Yeast cells were grown in YPD (1% yeast extract, 2% peptone, 3% glucose) as rich medium or YNB (0.68% yeast nitrogen base) supplemented with the required amino acids and 3% glucose as minimal medium. *ADH2* derepression was obtained as follows. Cells grown overnight to a 0.5 optical density unit at 600 nm per ml were washed twice with water and resuspended in the same volume of medium containing 0.05% glucose. Samples were collected at different times, and both chromatin and RNA analyses were performed.

Plasmid. ADR2-BS-pBR322 (74) was a kind gift of E. T. Young. This plasmid DNA was used to prepare probes for the indirect end-labeling analysis. It consists of a 2,250-bp genomic fragment, containing the entire *ADH2* gene, inserted in the pBR322 *Bam*HI site. For the ADH2 sequence, see reference 48a.

Enzymes. All nucleases and the Klenow enzyme were purchased from Boehringer Mannheim, *Taq* polymerase was purchased from Promega, and Zymolyase 100T was purchased from Seikagaku Corp.

Chromatin analysis. All methods are based on nuclease treatment of nystatin-permeabilized spheroplasts (70). Cells (30 to 50 optical density units per sample) were pelletted and resuspended in 10 ml of Zymolyase buffer (1 M sorbitol, 50 mM Tris-HCl [pH 7.5], 10 mM 2-mercaptoethanol) in the presence of 0.075 mg of Zymolyase 100T per optical density unit. Incubation was carried out for 30 min at 30°C. The pelletted spheroplasts were resuspended in nystatin buffer (50 mM NaCl, 1.5 mM $CaCl_2$, 20 mM Tris-HCl [pH 8.0], 1 M sorbitol, 100 μg of nystatin per ml) and divided into 0.25-ml aliquots. Micrococcal nuclease (MN) (1 to 10 U) or restriction endonucleases (20 to 80 U) were added to each aliquot of permeabilized spheroplasts, and incubation was performed at 37°C for 15 min (MN) or 30 min (restriction enzymes). The reaction was stopped with 1% sodium dodecyl sulfate (SDS)–5 mM EDTA (final concentrations). Proteinase K (40 μg per sample) was added, and the samples were kept at 56°C for 2 h. The DNA was then purified by phenol-chloroform extractions (three times) and ethanol precipitation.

(i) Low-resolution analysis. Indirect end-labeling analysis (77) was performed as follows. After treatment with the appropriate restriction enzymes, the samples were electrophoresed in 1.5% agarose–Tris-borate-EDTA (TBE) gels (1.75 V/cm), transferred to BA-S 85 nitrocellulose paper (Schleicher & Schuell), and hybridized by standard procedures.

(ii) High-resolution analysis. MN footprinting was performed as follows. After treatment with the appropriate restriction enzyme, the samples were primer extended with *Taq* polymerase in the presence of a 5′-end-labeled oligonucleotide. The primer extension products were then analyzed by electrophoresis on 6% polyacrylamide–TBE gels.

Nucleotide-level mapping of mononucleosome borders. The method used for nucleotide-level mapping of mononucleosome borders has been described previously (8, 9, 16, 69). Nystatin-permeabilized spheroplasts were treated with large amounts of MN (80 U/ml) for 15 min at 37°C, and the reaction was stopped with 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). After digestion with proteinase K in 0.2% SDS, the samples were extracted three times with phenol-chloroform and ethanol precipitated. Purified DNA was then electrophoresed on 1.5% agarose–TBE gels to isolate the mononucleosomal DNA. After electroelution, monomer DNA was partially labeled at low specific activity and run on denaturing polyacrylamide gels to remove internally nicked molecules. Single-stranded monomer DNA was eluted and primer extended with *Taq* polymerase in the presence of a labeled oligonucleotide, obtained by filling in with two radioactive nucleotides and Klenow enzyme. The primer extension products were then analyzed by electrophoresis on 6% polyacrylamide–TBE gels.

RNA analysis. Total RNA from yeast cells was prepared as described in reference 53. After spectrophotometric determination of the amount of RNA present, 10 μg from each sample was run in 1.2% agarose–morpholinepropanesulfonic acid (MOPS) gels (containing formaldehyde as a denaturing agent [19]). The gels were stained with ethidium bromide and photographed to visualize rRNA as a control for equal loading. Northern (RNA) analysis was performed by standard procedures, with Hybond N+ nylon paper (Amersham). For hybridization, 5′-end-labeled oligonucleotide 8 (see the map in Fig. 1) was used.

RESULTS

Nucleosome positioning on the *ADH2* promoter region under repressing conditions. (i) Low-resolution analysis. A schematic representation of the *ADH2* gene is given in Fig. 1. We used MN digestion of nystatin-permeabilized spheroplasts to identify the positions of the nucleosomes over the *ADH2* promoter region in the presence of 3% glucose. Indirect end-labeling analysis (77) allowed us to map the approximate position of eight nucleosomes. Figure 2 shows the results. (i) Nucleosome particles occupy defined positions over the *ADH2* promoter, as revealed by the presence of protection against MN cleavage (white areas defined by hypersensitive nucleosome borders). (ii) The protected areas have different lengths, the longest being those occupied by nucleosomes −1 and −2, although it cannot be excluded that these two large areas are due to protection by additional proteins. (iii) The region included between nucleosomes −1 and −2, containing the upstream activating sequences (UAS1 and UAS2, as defined in reference 80), is slightly too short to be compatible with the presence of a core particle; primer extension analyses were used to demonstrate the absence of nucleosome from this region (Fig. 5 shows that there is no protection of suitable length in the MN chromatin digest [lane R] compared with the deproteinized sample [lane C]); in addition, by using a couple of divergent oligonucleotides located in the middle of this area (same assay as in Fig. 3), we did not find elongation products on monomeric DNA produced by extensive MN treatment of spheroplasts, again indicating the absence of a monomeric particle protecting this region (data not shown). (iv) The TATA box and the RNA initiation sites are covered by nucleosomes −1 and +1, respectively (for a high-resolution mapping, see Fig. 3). (v) A poly(dA-dT) tract (20 residues) is covered by nucleosome −1, even though not in a central position.

The information obtained by the low-resolution mapping was used to program the oligonucleotides for the nucleotide-level localization of nucleosomes. For each nucleosome to be analyzed, two oligonucleotides which map in its center and are divergently directed were chosen (see the map in Fig. 3).

(ii) High-resolution analysis. To locate the precise boundaries of the relevant nucleosomes, we then applied a recently developed technique, which essentially consists of the use of primer extension analysis to identify the borders of mononucleosomes produced by extensive MN treatment of the samples (see Materials and Methods). This type of assay has already been applied by several different groups, both in vitro and in

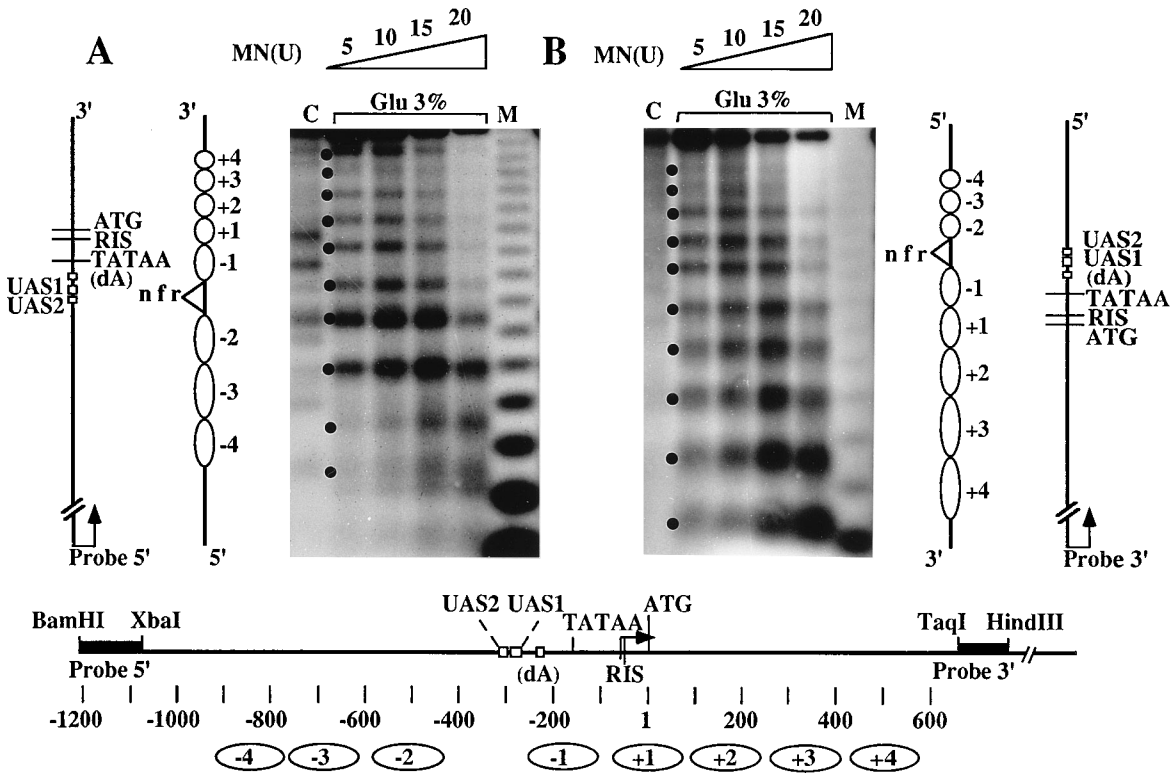

FIG. 2. Chromatin organization of the *ADH2* promoter region under repressing conditions (3% glucose): low-resolution mapping. Nystatin-permeabilized spheroplasts from CH335 cells grown in YP medium containing 3% glucose were reacted with increasing amounts of MN (U = units/0.25 ml), deproteinized, and digested with *Bam*HI and *Hin*dIII (map positions are −1202 and +760, respectively). The samples were electrophoresed through 1.5% agarose–TBE gels and transferred to a nitrocellulose membrane. The same filter was then hybridized with probe 5′ (A) or probe 3′ (B). Nucleosomes (i.e., protected areas) are represented as ovals. Dots indicate MN cleavages on nucleosome borders. Their positions (±20 bp) are as follows: (A) from top to bottom, +573, +398, +228, +73, −102, −282, −432, −602, −777, −892; (B) from bottom to top, +602, +440, +270, +100, −60, −270, −390, −610, −765, −920. Vertical map positions are only indicative. nfr, nucleosome-free region. Lanes C contain deproteinized chromosomal DNA reacted in vitro with MN and the same restriction enzymes as the in vivo samples. Lanes M contain size markers (123-bp ladder from Bethesda Research Laboratories).

vivo (7, 24, 28, 78). Figure 3 shows the results of this analysis for nucleosomes −2, −1, and +1, spanning the region considered to be essential for promoter activation (6). Each experimental panel is composed of (i) a full-length 150 ± 4-nucleotide monomer DNA purified and labeled (lanes N); (ii) a size marker (lanes M); (iii) the products of the primer extension from a centrally located oligonucleotide up to the in vivo MN-induced cleavage sites, which identify the nucleosomal borders (lanes B); and (iv) (only for the first two panels) ADR2-BS-pBR322 plasmid DNA treated in vitro with MN and reacted with the same oligonucleotide (lanes P). The identity of some of the in vivo nucleosome borders with in vitro MN cleavage sites is in agreement with the data presented in Fig. 2 (for example, both upstream and downstream borders of nucleosome −2 coincide with MN-sensitive sites). The borders are identified by symbols: matching positions from the two divergent oligonucleotides, whose distance approaches the monomer size (146 bp), have the same symbol. When the bands differ by only a few nucleotides, an average value has been assigned. Each of the three nucleosomes studied is characterized by a family of alternative positions (map in Fig. 3). In particular, the downstream borders of nucleosome −1 alternatively overlap with the upstream borders of nucleosome +1.

An interesting feature of this high-resolution mapping is the position of the −1 nucleosome upstream borders, located immediately adjacent to the UAS1 sequence, which is known to bind the activator Adr1p (55) (see Discussion).

Chromatin conformational changes during *ADH2* promoter activation. (i) Low-resolution analysis. The chromatin organization of the *ADH2* gene is kept in a repressed configuration in the presence of glucose. Thus, glucose seems to act negatively on the expression of this gene by rendering the relevant promoter elements inaccessible to the transcription machinery. We therefore asked whether any modification in the nucleosomal arrangement could be detected following a reduction of the amount of glucose in the medium (derepressing conditions). A kinetic analysis of the *ADH2* chromatin organization during promoter activation is shown in Fig. 4. Cells grown in YP medium containing 3% glucose were treated with 0 (lanes U, untreated sample) or 1 (lanes R, repressing conditions) MN unit/0.25 ml. After being washed twice with water, the cells were resuspended in the same volume of YP medium containing 0.05% glucose. Aliquots were withdrawn after 30 min and 1 and 3 h, and the chromatin, digested with 1 MN unit/0.25 ml, was analyzed by indirect end labeling (Fig. 4A and B). A small fraction of cells from each aliquot was used to analyze the RNA steady-state level (Fig. 4C). A chromatin conformational change is observed after 1 and 3 h of induction (Fig. 4A and B): intermediate bands appear in the organized pattern. Two of them correspond to the two most intense MN cleavage signals on naked DNA (lanes C, arrows): they are located at the level of the nucleosomes −1 and +1. Additional modifications are observed for nucleosomes −2 and +2, mainly after 3 h of induction. The pattern of modifications seen at 3 h of induction

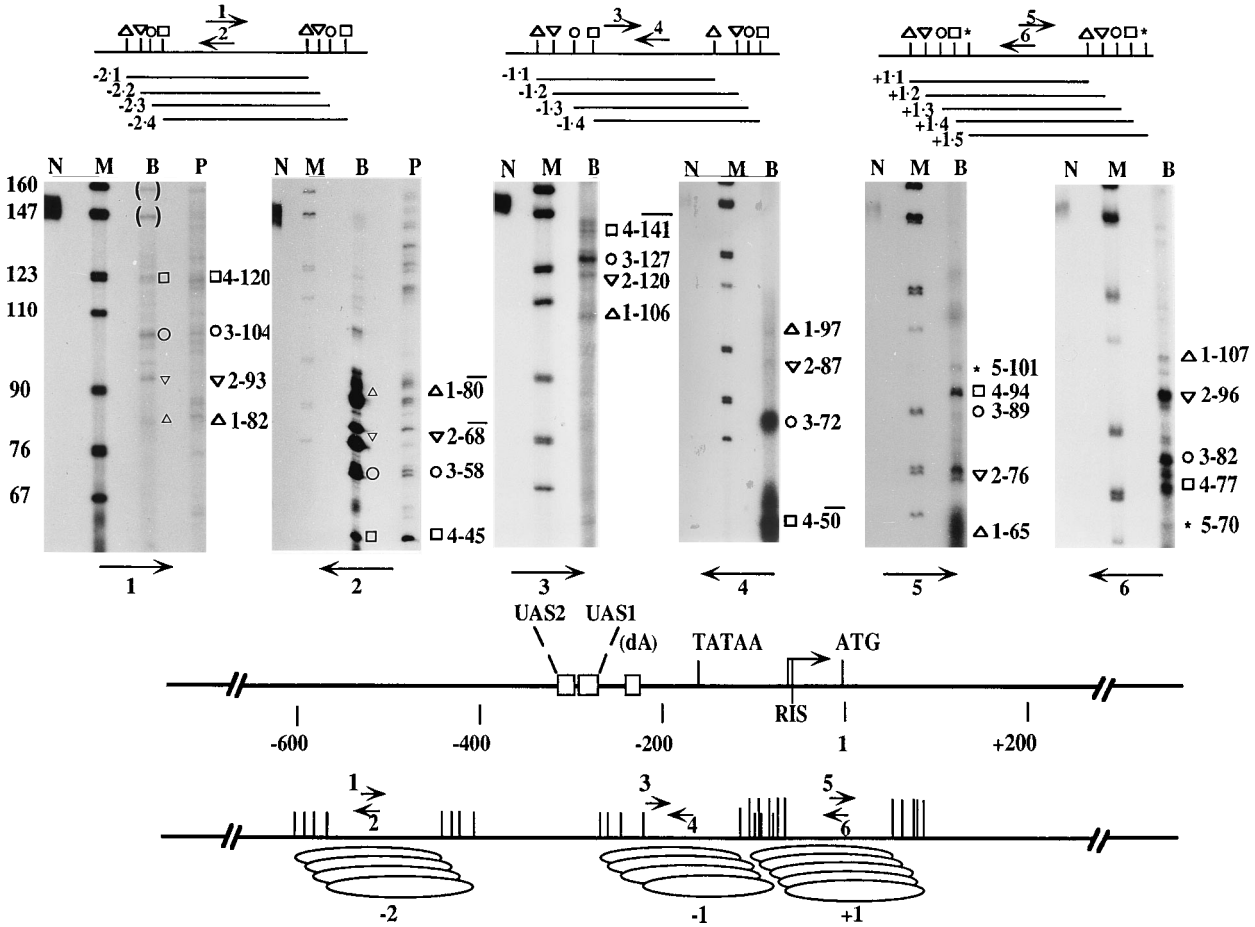

FIG. 3. High-resolution mapping of nucleosomes −2, −1, and +1 under repressing conditions (3% glucose). Lanes: N, monomer-sized (150 ± 4 bp) DNA fragments, produced by extensive MN treatment of nystatin-permeabilized spheroplasts from CH335 cells grown in YP medium containing 3% glucose; M, size markers (pBR322 DNA digested with *Msp*I); B, *Taq* polymerase elongation products from the oligonucleotide indicated at the bottom of each panel (map positions in Fig. 1). Oligonucleotides 1 and 2, 3 and 4, and 5 and 6 were used to map the borders of nucleosomes −2, −1, and +1, respectively. Odd-numbered oligonucleotides define the downstream borders; even-numbered oligonucleotides define the upstream ones. The logic of this mapping is described in detail in reference 16. The borders are identified by the values reported at the right side of each panel. The positions of the protected DNA segments are determined by summing couples from the two sides, subtracting the distances between the origins of the two divergent oligonucleotides, and choosing the values closest to the monomer size (146 bp). The subtraction factors are as follows: 8 for oligonucleotides 1 and 2, 49 for 3 and 4, and 16 for 5 and 6. For example, for nucleosome −2.4, we couple (120 + 45) − 8 = 157 bp; for nucleosome −1.1, we couple (106 + 97) − 49 = 154 bp; for nucleosome +1.2, we couple (76 + 96) − 16 = 156 bp, etc. The map positions of the nucleosome borders are as follows (from top to bottom): primer 1, −406, −422, −433, and −444; primer 2, −599, −587, −577, and −564; primer 3, −78, −92, −99, and −113; primer 4, −267, −257, −242, and −220; primer 5, +89, +82, +77, +64, and +53; primer 6, −103, −92, −78, −73, and −66. Lanes P, ADR2-BS-pBR322 DNA treated with MN in vitro and reacted with the oligonucleotide indicated at the bottom of the panel. The full-length (146 ± 2 bp) elongation product occasionally observed (parentheses in the left-hand panel) might be due to one of several possible artifacts and was not considered further.

does not change by 5 h, when the steady-state mRNA level is only slightly higher (less than twofold [results not shown]). The same experiment was also performed with larger amounts of MN, but the intensity of the hypersensitive bands turned out to be lower (results not shown).

Thus, release from glucose repression involves destabilization of two specific nucleosomes in the *ADH2* promoter, the ones covering the TATA box and the RNA Initiation Sites.

(ii) MN footprinting of nucleosome −1. To understand the nature of the chromatin rearrangement described above, we have used a footprint analysis to examine at high resolution the structure and variations of nucleosome −1, occupying the most important promoter element: the TATA box. This assay consists of in vivo digestion of chromatin followed by primer extension of the purified material (8, 69). When a single-copy gene has to be studied, as in this case, very long exposures of the autoradiograms are required and the background has to be low (as shown in Fig. 5, lane U). Samples treated as in Fig. 4 but with 10 MN units/0.25 ml were primer extended with *Taq* polymerase from 5′-end-labeled oligonucleotide 7 (map in Fig. 1). The sites of MN cleavage on naked DNA are shown in Fig. 5, lane C. Under repressing conditions (lane R, 3% glucose), a clear protection of the MN-sensitive sites is observed. This region corresponds to the protection of the area of common occupancy by the alternative members of the family of nucleosome −1, described in the legend to Fig. 3. After the amount of glucose in the medium was reduced, a gradual loss of protection was observed for all but one of the MN-sensitive sites (see Discussion).

The nucleosome-free region mapped in Fig. 2 does not show any significant change in MN sensitivity during promoter activation.

The kinetics of destabilization of nucleosome −1 do not correlate with mRNA accumulation: analysis of the accessibility to *Sph*I restriction enzyme under derepressing conditions. One important issue to be addressed in the process of *ADH2*

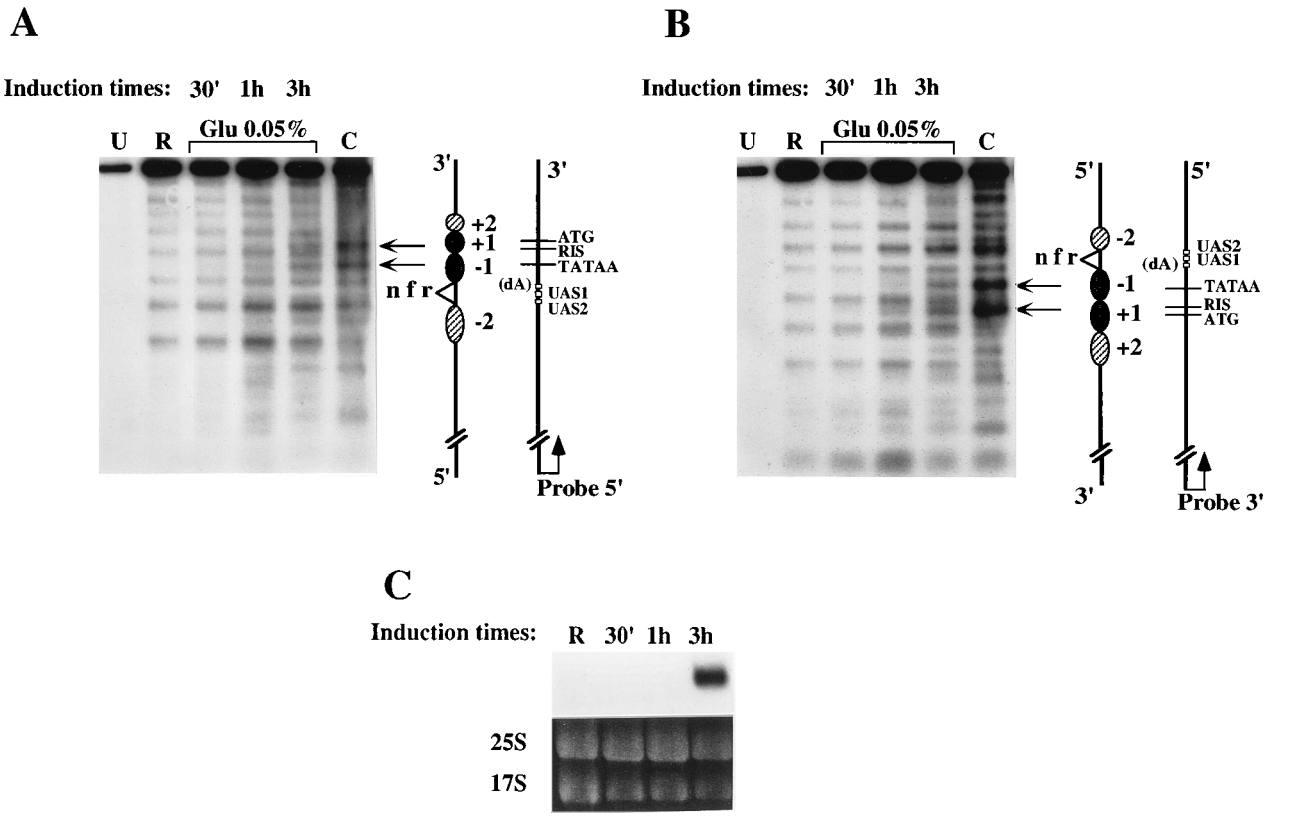

FIG. 4. Chromatin conformational changes during *ADH2* promoter activation: low resolution analysis. (A and B) Nystatin-permeabilized spheroplasts from CH335 cells grown in YP medium containing 3% glucose were treated with 0 (lanes U, untreated sample) or 1 MN unit/0.25 ml (lanes R, repressed). After being washed twice with water, the samples were resuspended in the same volume of YP medium containing 0.05% glucose. At the indicated times, aliquots were withdrawn and reacted with 1 MN unit/0.25 ml, and indirect end-labeling analysis was performed. *Bam*HI and *Hin*dIII were used to digest all the samples. The same nitrocellulose filter was hybridized with probe 5′ (A) or probe 3′ (B). Lanes C contain deproteinized chromosomal DNA treated in vitro with MN. Arrows indicate the most intense MN cleavage sites, which become accessible at increasing induction times: their map positions (±20 bp) are −165 and −26. Rearranged nucleosomes are indicated by stippled (−1 and +1) or hatched (−2 and +2) ovals. (C) Northern blot analysis of *ADH2* mRNA during derepression. Total RNA was prepared from a small aliquot of the same cells used for chromatin analysis. As a probe we used 5′-end-labeled oligonucleotide 8 (map in Fig. 1).

promoter activation is the time-dependent nature of the chromatin conformational modification. Is the loss of nucleosomal protection for the promoter elements a prerequisite for or a consequence of transcription?

We looked at the structure of nucleosome −1 during *ADH2* promoter activation by using restriction endonucleases as chromatin probes. These enzymes can recognize and cleave their target sites in chromatin DNA only if they are correctly oriented on the nucleosome surface or if they occur in nucleosome free regions. In this regard, this analytical tool can be more specific and sensitive than MN (whose patterns are always complicated by the ratio between the kinetics of digestion and the distance from the probe used for mapping). Initially, we used *Alu*I because of its multiple presence in several important locations of the *ADH2* promoter: unfortunately, it turned out to be able to cut even under repressed conditions (results not shown). The next choice was *Sph*I, whose cut site maps 10 nucleotides upstream of the TATA box. Figure 6 shows the results of this type of analysis: nystatin-permeabilized spheroplasts were treated with 20 and 60 *Sph*I units/0.25 ml under repressing conditions (lanes R) and after 30 min and 3 h of induction (in YP medium containing 0.05% glucose, as indicated). The in vitro-treated chromosomal DNA sample (lane C) shows three bands: only the most intense one (at the bottom of the gel) corresponds to the expected cleavage event. The other two bands were not predicted from the sequence data and probably derive from *Sph*I star activity (on sites which are only partially homologous, identified by us on the sequence but not analyzed further).

The relevant result is the strong accessibility of the standard *Sph*I site, already visible after 30 min of derepression, when the transcription is not yet detectable (Fig. 4C). The cleavage rate increases only slightly (less than twofold) when the transcription level is already high (i.e., at 3 h). The *ADH2* mRNA signal peaks, for this strain, at about 4 h from induction (data not shown) (19).

Therefore, destabilization of nucleosome −1 occurs very soon after the amount of glucose in the medium is reduced. In addition, the relative increase of the accessibility of the *Sph*I site does not correlate with the amount of mRNA accumulated. This suggests (but does not formally prove) that the chromatin conformational change is likely to be a prerequisite for transcription and is not dependent on the process itself.

Glucose requirement for chromatin remodeling in spheroplasts from cells grown in minimal medium. All the experiments described so far were performed with rich medium (YP medium). We tried to reproduce them with minimal medium (YNB medium plus the required amino acids) and found that to detect the nucleosome rearrangement under derepressing conditions, the spheroplasts had to be incubated in the presence of a small amount of glucose (0.05%). These data are presented in Fig. 7A. Cells grown in YNB medium containing

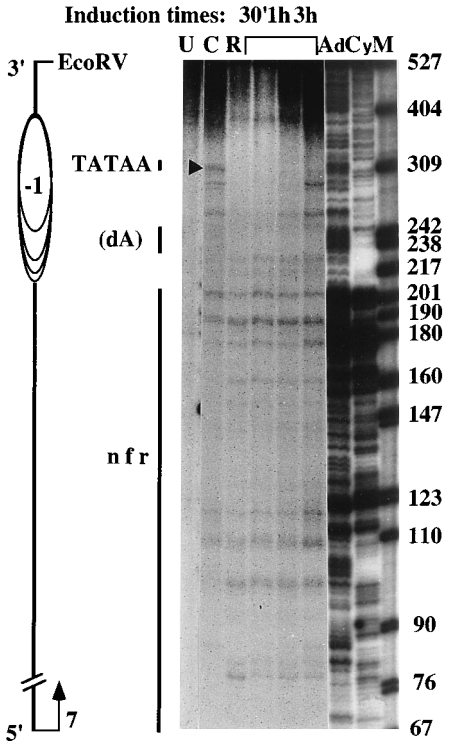

FIG. 5. MN footprinting of nucleosome −1. Nystatin-permeabilized spheroplasts from CH335 cells grown in YP medium containing 3% glucose were treated with 0 (lane U, untreated sample) or 10 (lane R, repressed) MN units. After being washed twice with water, the samples were resuspended in the same volume of YP medium containing 0.05% glucose. At the indicated times, aliquots were withdrawn and reacted with 10 MN units, and the purified DNA was primer extended with *Taq* polymerase from 5′-end-labeled oligonucleotide 7 (map in Fig. 1). *Eco*RV was used to digest all the samples. Other lanes: C, deproteinized chromosomal DNA treated in vitro with MN; Ad and Cy, adenine and cytosine sequencing lanes, obtained from ADR2-BS-pBR322 plasmid DNA primer extended with Taq polymerase as above; M, size markers, pBR322 DNA digested with *Msp*I.

3% glucose were washed twice and resuspended in the same volume of YNB medium containing 0.05% glucose. At the indicated times, aliquots were withdrawn, pelletted, resuspended in Zymolyase buffer, and divided into two samples, the only difference being the addition to one sample of 0.05% glucose in both the Zymolyase and the nystatin buffers (the last one used for MN treatment). For the spheroplasts complemented with the small amount of glucose (lanes +0.05% Glu), the expected destabilization of nucleosomes −1 and +1 is visible; MN accessibility increases slightly with time, and at 3 h of induction, nucleosome −2 is also destabilized (exactly as observed when YP medium was used). However, a chromatin pattern resembling the repressed state is visible for the spheroplasts without glucose at 45 and 90 min of induction (lanes −0.05% Glu). Only after 3 h did we observe the expected chromatin transition. The Northern analysis shows the same kinetics of *ADH2* mRNA accumulation for both groups of samples (Fig. 7B). The aliquots used for the RNA analysis were withdrawn from the spheroplasted samples resuspended in nystatin buffer, just before the addition of MN, to ensure that the glucose difference was not influencing *ADH2* transcription.

Therefore, the spheroplasts from cells grown in minimal medium require the addition of a nutrient (glucose) to remain active in terms of dynamic chromatin change, at least for the first 90 min of induction (see Discussion).

Adr1p is required for the nucleosome conformational changes. What is the nature of the trigger in the process of nucleosome destabilization? Once the glucose repression starts to weaken, an *ADH2*-specific factor(s) could become available to drive promoter activation. Among the various candidates, the protein encoded by the *ADR1* gene (Adr1p) is the only factor known to bind directly to the *ADH2* promoter. Its binding sites in the UAS1 (12, 21) are located in the nucleosome-free region, very close to the nucleosome −1 upstream borders (map in Fig. 3). By using a mutant strain in which the *ADR1* gene is disrupted, we tested whether Adr1p is required for the chromatin conformational change observed. The results are reported in Fig. 8. A comparison of the *ADH2* nucleosome organization between isogenic wild-type and *adr1* mutant strains under repressing conditions (3% glucose) shows identical patterns (Fig. 8A). In contrast, under derepressing conditions (0.05% glucose), cells lacking Adr1p do not show any of the chromatin modifications found in the wild-type cells (Fig. 8B). Residual *ADR1*-independent transcription (10- to 20-fold lower level than in the wild-type strain) is observed by Northern analysis (Fig. 8C).

This result is consistent with the need for Adr1p binding to UAS1 to trigger the series of events leading to nucleosome destabilization.

DISCUSSION

Chromatin organization of the inactive *ADH2* promoter. We have described the nucleosome distribution over the *ADH2* promoter region at both low and high resolution, under repressing conditions. As for many other genes analyzed, nucleosomes occupy defined positions with respect to the underlying DNA sequences. The relevant results of the low-resolution MN analysis are (i) the presence of a nucleosome free region containing the UAS1 and UAS2; (ii) the protection of the TATA box and of the RNA initiation sites exerted by nucleosomes −1 and +1, respectively; and (iii) the inclusion of a 20-bp poly(dA-dT) tract in the DNA wrapped around nucleosome −1.

The nucleotide-level determination of the nucleosome −2, −1, and +1 borders shows that each particle is characterized by a family of potentially and partially overlapping positions. The presence of multiple overlapping positions is not a new phenomenon. A similar situation has been observed in other yeast gene systems in vivo (9, 16, 69). This could be due to the absence of histone H1 in this eukaryote. In addition, multiple borders have been reported for other eukaryotic systems, both in vitro and in vivo (26, 73, 78). For the *ADH2* promoter, the proximity of the downstream borders of nucleosome −1 and the upstream borders of nucleosome +1 is intriguing, since they rearrange simultaneously during activation. A functional significance for that particular arrangement could therefore be the possibility of driving a coordinate remodeling for "communicating" nucleosomes.

The existence of the nucleosome-free region, indicated by the low-resolution mapping, has been confirmed by the footprinting data. This open region, containing UASs, is reminiscent of the organization found in other two well-characterized yeast gene systems, the *GAL1-GAL10* intergenic region (23) and the *PHO5* promoter (3): in the *PHO5* system, the binding sites for the activating protein Pho4 are in part accessible and in part covered by nucleosomes (71). For the *ADH2* promoter, the two binding sites for Adr1p (12, 21) are adjacent to the upstream borders of nucleosome −1 and apparently accessible. In particular, the binding affinity of the monomer which has to occupy the right half of the UAS1 sequence could be

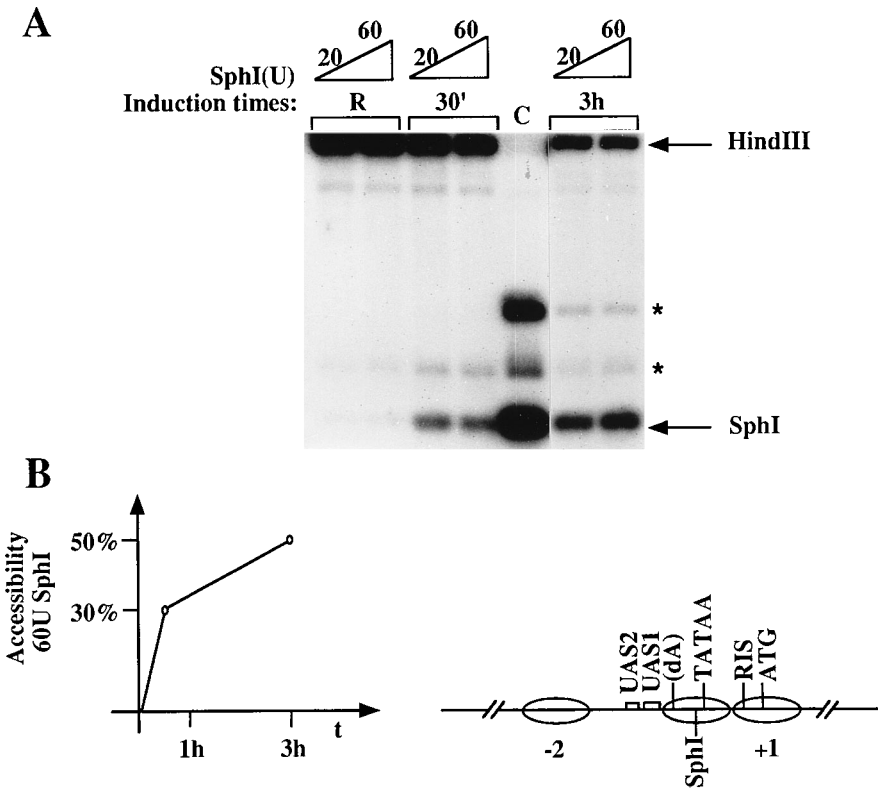

FIG. 6. *Sph*I restriction enzyme accessibility. (A) Nystatin-permeabilized spheroplasts from CH335 cells grown in YP medium containing 3% (lanes R) or 0.05% glucose for 30 min (lanes 30′) or 3 h (lanes 3h) were reacted with 20 and 60 *Sph*I units/0.25 ml, purified, and treated with *Bam*HI and *Hin*dIII. Probe 3′ (map in Fig. 1) was used for hybridization. The band derived from the unique cleavage site predicted by the sequence data is indicated by the arrow (*Sph*I) at the bottom of the gel. Two unpredicted bands ("pseudosites") are marked by asterisks. Their approximate positions are −417 (lower) and −717 (upper). Lane C, deproteinized chromosomal DNA treated in vitro with *Sph*I and *Hin*dIII. (B) Graph showing the *Sph*I relative accessibility measured with 60 U of enzyme. Densitometer scanning was used for quantitation.

increased or decreased, depending on the specific position of the nucleosome borders in that cell population. An in vivo detailed Adr1p footprinting under repressing and/or activating conditions is still lacking. This analysis is necessary to clarify the exact relationship between Adr1p and nucleosome −1.

A closer inspection of the mononucleosome border data reveals that a homogeneous stretch of 20 adenines is wrapped around nucleosomes −1.1, −1.2, and −1.3, although not in a central position. Its location between the UAS1 and the TATA box sequences suggests a function for this potentially nucleosome-destabilizing structure (72). This function does not necessarily consist of facilitating nucleosome removal, since it has been reported that nucleosomes do actually form in vitro on poly(dA-dT) stretches (36, 44). The role of poly(dA-dT) sequences should still be considered a controversial matter (31, 49, 50). Interestingly, two constitutive up-promoter mutants, characterized by a doubling of this poly(dA-dT) tract, have been isolated (48).

Chromatin conformational change during ADH2 activation. The *ADH2* nucleosome-mapping data for cells grown under repressing conditions show that glucose exerts its inhibitory effect by keeping the relevent promoter sequences (TATA box and RNA initiation sites) in a nucleosomal configuration, thus precluding their engagement with the transcription machinery. When this nutrient becomes limiting in the culture medium, several signal transduction pathways are activated to lead this message to the nucleus (27, 46). We searched for nuclear events occurring at the *ADH2* promoter level in the presence of low glucose levels and found that two specific nucleosomes are indeed perturbed. The time-dependent loss of protection from MN cleavage, shown by both low- and high-resolution analyses, can be explained by (i) the complete displacement of nucleosomes −1 and +1 in an increasingly large fraction of cells or (ii) the gradual disruption of the conformation of the examined nucleosomes. The retention of protection in the presence of low levels of glucose, for the site indicated by the arrowhead in the −1 nucleosome footprint (Fig. 5), does not provide a conclusive indication. That site maps in correspondence to the TATA box: the presence in that region of the transcription initiation complex could protect the DNA from MN attack, even in the presence of complete histone displacement. Nevertheless, we favor the second explanation for several reasons: (i) quite a large amount of energy would be required to overcome the entire set of histone-DNA interactions; (ii) the chromatin switches back to the repressed state very rapidly during spheroplast formation (Fig. 7); and (iii) most of the evidence presented so far on chromatin remodeling is compatible with the retention of the nucleosomal configuration, even in the presence of substantial change in nuclease accessibility (discussed in references 1 and 59).

Chromatin remodeling and transcription. What is the exact cause-effect relationship between these two events? In vitro studies on nucleosome assembled templates indicate that the binding of factors to their DNA targets when wrapped around a nucleosome is sufficient to drive a change in the particle structure, even in the absence of transcription (66, 73). This

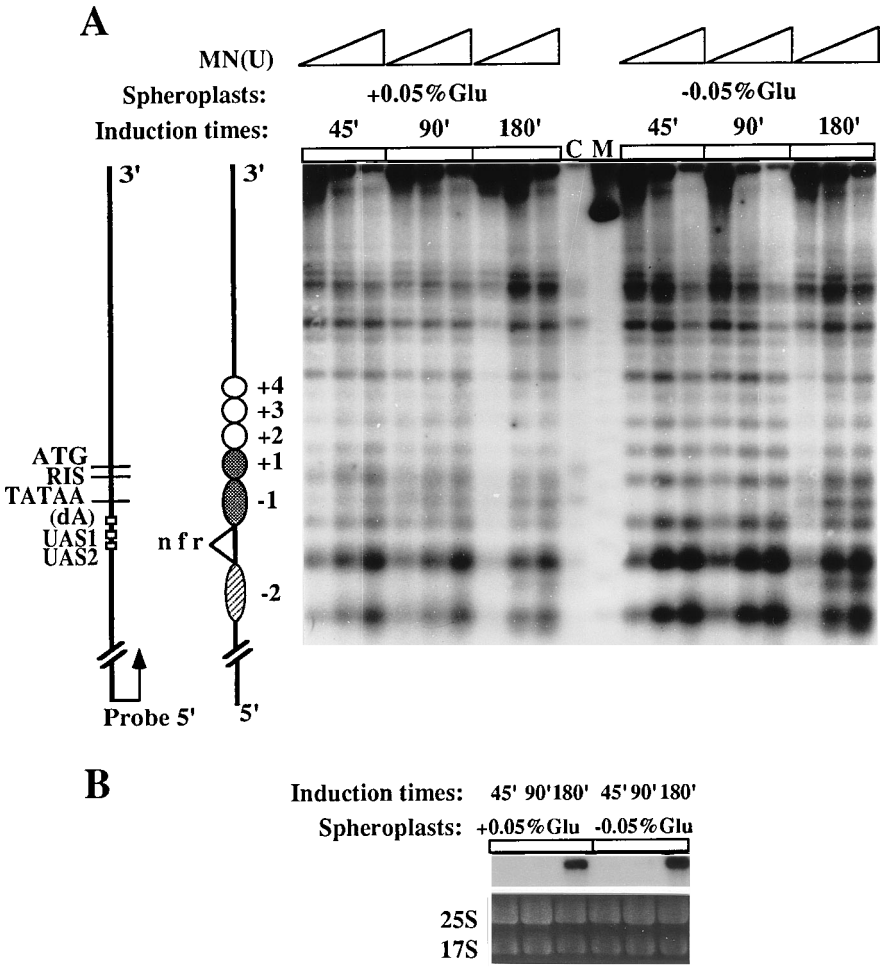

FIG. 7. Glucose requirement for chromatin-remodeling detection. (A) CH335 cells were grown in YNB medium containing 3% glucose, washed twice with water, resuspended in the same volume of YNB medium containing 0.05% glucose, and incubated for 45, 90, or 180 min. At the indicated times, aliquots were withdrawn and the chromatin analysis (spheroplast preparation and digestion with 5, 10, and 15 MN units/0.25 ml) was performed in the presence or absence of glucose (±0.05%Glu). *Bam*HI was used to digest all the samples. Lanes: C, deproteinized chromosomal DNA treated in vitro with MN; M, size markers (123-bp ladder from Bethesda Research Laboratories). (B) Northern analysis of *ADH2* mRNA during derepression. Total RNA was prepared from a small aliquot of the same nystatin-permeabilized spheroplasts used for chromatin analysis. As a probe, we used 5′-end-labeled oligonucleotide 8 (map in Fig. 1).

event can, in turn, allow the formation of additional cooperative protein-DNA interactions as shown both in vitro and in vivo (2, 4, 60, 65). In vivo analysis of yeast TATA box mutants, for promoters whose activation is accompanied by chromatin remodeling, demonstrates that nucleosomes change structure under derepressing conditions even when transcription is impaired (5, 22, 33). Evidence of transcription-induced chromatin change has been provided for one heat shock promoter in *S. cerevisiae*, although the modifications occur at the 3′ end of the gene (34).

We think that for the *ADH2* promoter also, the nucleosome destabilization represents a prerequisite for (rather than a consequence of) transcription, because of the following observations: (i) the kinetics of nucleosome disruption, studied by using both MN (Fig. 4) and *Sph*I (Fig. 6), do not correlate with the kinetics of mRNA accumulation (for example, the accessibility of the *Sph*I site is already very high after 30 min of induction, when the *ADH2* mRNA signal is not yet visible, and increases only slightly with time, as opposed to the severalfold increase in transcription [Fig. 6]); (ii) when the chromatin rearrangement cannot be detected, because of inert spheroplasts in the absence of glucose, the *ADH2* transcription is not influenced, suggesting that the two events are independent (Fig. 7); (iii) transcription-dependent chromatin changes are shown by nucleosomes −2 and +2, whose destabilization is revealed after 3 h of induction (Fig. 4).

Dynamic versus inert chromatin configurations. Nucleosomes tend to remain stable in their expected locations, on the basis of the ensemble of translational and rotational signals intrinsic to the nucleotide sequence (64). Even when the positions are not unique but multiple and alternative, core particles select the most energetically favorable locations (9). The chromatin repressed state is the rule for the *ADH2* promoter: this state cannot be easily perturbed. When the gene has to be activated, energy must be put into the system to overcome nucleosome-exerted repression. This counteraction is likely to be a dynamic process that must be actively maintained. It was reported previously (52) that spheroplast preparation may be detrimental to chromatin analysis when studying the activation process: apparently, nucleosomes rapidly switch back to the inactive configuration, maybe because the nutrients (i.e., the energy sources) are washed out. This could explain why the spheroplasts from cells grown in minimal medium and low levels of glucose have to be complemented with a small amount

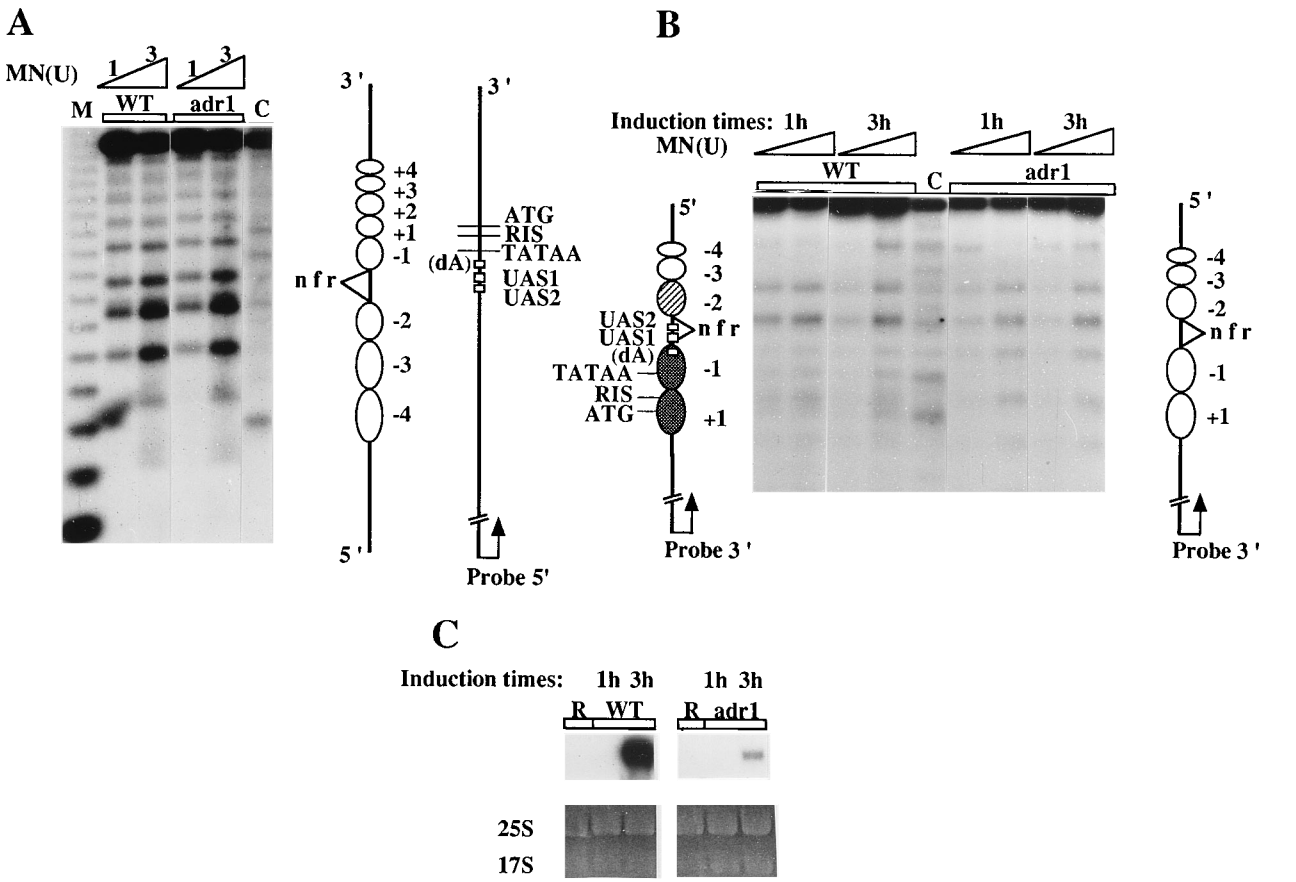

FIG. 8. Chromatin analysis of an *ADR1*-disrupted mutant strain. (A) Indirect end-labeling analysis of two isogenic strains (HD93-15D [WT] and HD93-15DdB [*adr1*]) under repressing conditions (3% glucose). Samples were digested with *Bam*HI and *Hin*dIII. Probe 5′ was used for the hybridization. (B) Nystatin-permeabilized spheroplasts from cells grown under derepressing conditions (YNB medium containing 0.05% glucose) were treated with 3 and 6 MN units/0.25 ml at the indicated induction times, in the presence of 0.05% glucose. Lane C contained deproteinized chromosomal DNA treated in vitro with MN. Samples were digested with *Bam*HI and *Hin*dIII. Probe 3′ was used for the hybridization. Rearranged nucleosomes (only for the wild-type [WT] strain) are shown as stippled ovals. (C) Northern analysis of *ADH2* mRNA in wild-type (WT) and *adr1* strains. R, repressing conditions (3% glucose).

of glucose to allow detection of chromatin remodeling (Fig. 7). This is true at least up to 90 min after induction. Interestingly, at 3 h of induction, even without complementation, nucleosomes appear destabilized. One possible explanation is that the cells have accumulated enough energy to keep the chromatin open even during spheroplast formation. Alternatively, in this dynamic process, there is some point (high transcription?) at which the equilibrium is strongly shifted toward the open configuration. Spheroplasts prepared from cells grown in rich medium seem to have fewer problems: we noticed, however, that addition of the same small amount of glucose to these spheroplasts substantially increases the MN accessibility signals (data not shown). In the above-mentioned study (52), *PHO5* chromatin remodeling was similarly linked to a glucose requirement. Whether the reason for this requirement involves ATP, as shown in some in vitro systems (66, 68, 73), has yet to be determined.

Adr1p-activating function. The only DNA-binding factor known for the *ADH2* promoter is the well-characterized Adr1p (14, 15, 18). Since this protein has been identified as a positive regulator and the *ADH2* promoter is negatively repressed by nucleosomes, we asked whether Adr1p has anything to do with the chromatin modifications we have observed. By using an *ADR1*-disrupted mutant, we have shown that Adr1p is indeed required for nucleosome destabilization (Fig. 8). Since its binding sites are located in the immediate vicinity of nucleosome −1 upstream borders, it is tempting to speculate that the function of this activator would be to trigger the remodeling of the nucleosome blocking the TATA box. When the distance between the Adr1p-binding sites and the TATA box is significantly increased, by the presence of a Ty element, *ADH2* transcription becomes *ADR1* independent: in this case, however, the dependence on the SWI-SNF complex becomes relevant (13). It is possible that this protein complex helps Adr1p in mediating nucleosome destabilization under normal conditions and becomes strongly required when the activator is too far from its usual location. Recently, several other genes whose expression is *ADR1* dependent have been discovered serendipitously (56), and many more appear to be potential targets for Adr1p (12). The interesting aspect here is that the distance between the UAS1 homologs and the TATA element is important for determining whether the homolog has enhancer activity: this distance is compatible with the presence of a nucleosome (110 bp for the *ADH2* promoter and 30 to 120 bp for the *CTA1* and *FOX2* genes [12]).

The very fact that the same activator is used in different systems will help to clarify whether the function of Adr1p is indeed to participate in overcoming the nucleosome repression.

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