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cAMP-dependent phosphorylation and inactivation of yeast transcription factor ADR1 does not affect DNA binding

(protein kinase/transcriptional activation/CYR1/ADH2/zinc finger)

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ABSTRACT **Transcription factor ADR1 increases the level of *ADH2* gene expression 200-fold by binding to a palindromic upstream activation sequence (UAS1) in the glucose-repressible *ADH2* promoter in *Saccharomyces cerevisiae*. cAMP-dependent protein kinase (cAPK) phosphorylates ADR1 *in vitro* and a yeast strain with elevated cAPK activity inhibits the ability of ADR1 to activate *ADH2* transcription *in vivo* [Cherry, J. R., Johnson, T. R., Dollard, C., Schuster, J. R. & Denis, C. L. (1988) *Cell* 56, 409–419]. Intact ADR1 protein was detected at comparable levels in extracts made from repressed or derepressed yeast cells, indicating that glucose repression is not due to absence of ADR1. ADR1 in extracts made from glucose-repressed and -derepressed cells bound UAS1 DNA with similar affinities despite having greatly different abilities to activate *ADH2* gene expression *in vivo*. A mutant form of ADR1 encoded by *ADR1-5^c^*, which has an altered consensus sequence for phosphorylation by cAPK conferred constitutive expression on *ADH2* but bound DNA to the same extent as wild-type ADR1 protein. Similarly, normal DNA binding was seen for ADR1 produced in mutants with altered levels of cAPK activity. Because inactivation of ADR1 by phosphorylation has no detectable effect on either DNA binding or ADR1 levels, ADR1 probably binds to UAS1 constitutively and phosphorylation prevents it from promoting transcription.**

Gene expression can be regulated by phosphorylation of DNA-binding transcription factors in response to an extracellular signal mediated by cAMP-dependent protein kinase (cAPK) (1). For instance, transcription of several mammalian neuroendocrine genes is activated by cAMP-dependent phosphorylation of the cAMP-responsive element binding protein (CREB), which binds at the cAMP-responsive elements (CRE) in regulated promoters (2, 3). In *Saccharomyces cerevisiae*, growth on glucose induces a cAMP signal that initiates a cascade of protein phosphorylation by cAPK (4–6), resulting in increased glycolysis and inactivation of several enzymes involved in gluconeogenesis (7, 8). Phosphorylation of transcription factors by cAPK may also regulate transcription of yeast genes for polyubiquitin (*UBI4*) (9), catalase I (*CTT1*) (10), and alcohol dehydrogenase II (ADHII) (*ADH2*) (ref. 11; K. Dombek, personal communication).

Transcription of the glucose-repressible *ADH2* gene encoding ADHII is activated 200-fold (derepressed) in glucose-free medium (12). Glucose repression of *ADH2* may be mediated in part by posttranslational modification of the constitutively expressed transcriptional activator ADR1 (13–15), rather than by a DNA-binding repressor (16). Two "zinc-finger" structures located between amino acids 100 and 160 in ADR1 (17, 18) are required for activation of *ADH2* transcription (19) and for DNA binding to a 22-base-pair (bp) upstream activation sequence (UAS) with dyad symmetry, UAS1 (20, 21), located 137 bp upstream from the "TATA box" in the *ADH2* promoter (22). UAS1 activates gene expression synergistically with an adjacent 25-bp sequence, UAS2, which is regulated independently of ADR1 (23).

Yeast ADR1 is a serine phosphoprotein whose activity appears to be negatively regulated by cAPK-dependent phosphorylation. Transcription directed by ADR1 binding at UAS1 becomes constitutive, partially escaping glucose repression in the *cyr1-2* mutant having temperature-sensitive adenylate cyclase (K. Dombek, personal communication). This mutant was shown (4) to produce very low levels of cAMP and thus to have low cAPK activity. Conversely, ADR1 activity is inhibited in the *bcy1* mutant, which lacks the regulatory subunit of cAPK, and thus has elevated cAPK activity (11). ADR1 contains several potential sites for phosphorylation by cAPK, including a recognition sequence (24), Arg-Arg-Ala-Ser-Phe, having the phosphoacceptor serine at residue 230 (17). Constitutive mutations that relieve glucose repression of *ADH2 in vivo* (13) alter this consensus site in ADR1 in a manner predicted to decrease phosphorylation of Ser-230 by cAPK (11).

Because cAPK appears to regulate glucose repression of *ADH2* by phosphorylating and inactivating ADR1, we addressed the question of whether phosphorylation inhibits DNA binding of ADR1 or modulates activation of transcription.

MATERIALS AND METHODS

Yeast Strains, Transformation, and Growth. Strains of *Saccharomyces cerevisiae* used in this study were 521-6Δ1 (*MATa adh1-11 adh3 adr1-Δ1::LEU2 leu2 trp1 ura1*), XV617 (*MATa ste5 his6 leu2 trp1 ura3*), CMY-215 (*MATa ura3-52 trp-1 his3-200 lys2-801a ade2-100° can1*) from Carl Mann (Department of Genetics, University of Washington, Seattle), CM1-2 (congenic to CMY-215 plus *leu2 cyr1-2^ts^*), and KD100-13 [isogenic to CMY-215 with *bcy1* disrupted with *sra1::URA3* (6)]. Transformation with plasmid DNA (25), growth and derepression of cultures, preparation of protein extracts, and ADH activity measurements have been described (16).

Plasmids Allowing ADR1 Overproduction in Yeast. Plasmid YEp1-ADR1 (Fig. 1*A*) was made by cloning the *ADR1* gene contained on a filled-in 6-kilobase (kb) *Nci* I DNA fragment of plasmid YEp1323ADR1 (17) into the filled-in *Eco*RI site of yeast expression vector pYcDE2, derived from pMA56 (26), such that the 120-bp truncated mRNA leader for *ADR1* was fused with the strong ADH1 promoter. YEp1-ADR1-5^c^ was made similarly using a 6-kb *Nci* I fragment from the *ADR1-5^c^* gene in YEpADR1-5^c^, which was made by placing a 4-kb *Nru*

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Abbreviations: cAPK, cyclic AMP-dependent protein kinase; UAS, upstream activation sequence; ADH, alcohol dehydrogenase.

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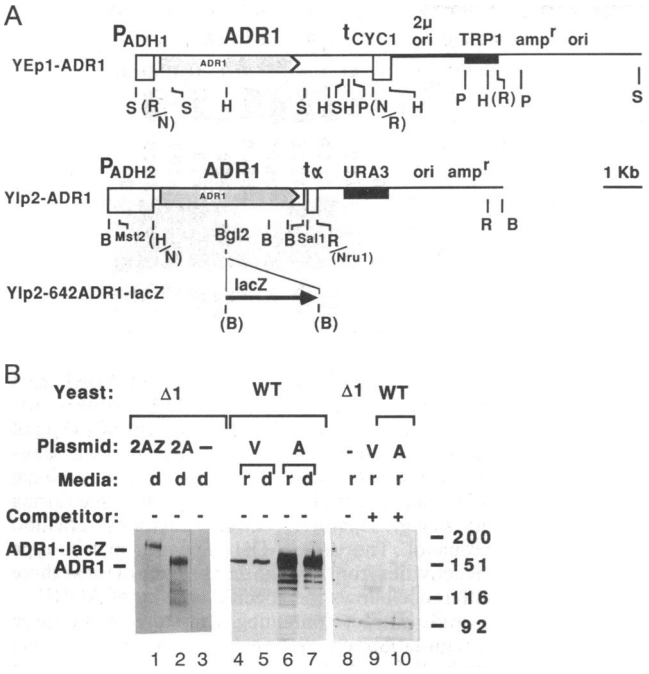

FIG. 1. Identification of ADR1 protein in yeast. (*A*) Linear plasmid maps are shown for YEp1-ADR1 (plasmid A) in which the *ADH1* promoter (P_{ADH1}) is fused with the *ADR1* gene in a yeast episomal (2μ) plasmid with the *TRP1* selectable marker; and for YIp2-ADR1 (plasmid 2A), in which the promoter for *ADH2* (P_{ADH2}) is fused with the *ADR1* gene in a yeast integrative plasmid with the *URA3* marker. Plasmid YIp2-ADR1 is similar to YIp2-642ADR1-lacZ (plasmid 2AZ), which contains *ADR1* fused at codon 642 with *lacZ* as indicated. The parental non-ADR1-containing plasmid to YEp1-ADR1 is pYcDE2 (plasmid V). B, *Bam*HI; S, *Sph* I; H, *Hin*dIII; R, *Eco*RI; N, *Nci* I; P, *Pst* I. Destroyed sites are in parentheses and *Eco*RI sites in *ADR1* are not included. (*B*) Western immunoblot of ADR1 in yeast extracts. Yeast protein extracts were prepared from strains grown in selective medium with glucose repression (lanes r) or after shifting for 10 hr to derepressing medium (lanes d). Strains 521-6-Δ1 (yeast Δ1) and CMY215 (yeast WT) were used. After cell breakage with glass beads, proteins were separated by SDS gel electrophoresis and immunoblotted with anti-ADR1 antisera. Competitor refers to a purified TrpE-ADR1 polypeptide used to raise the antiserum to ADR1. –, No competitor; +, 20 μg of competitor was incubated with the antiserum for 2 hr before adding the Western blot.

I–*Bgl* II fragment of YRp7-ADR1-5^c-23A (27) into YEp1323 ADR1. Plasmid YIp2-ADR1 was made by cloning the *ADH2* promoter fused with the ADR1 gene into the integrative vector YIp5 (called pBC72; ref. 28) and is shown in Fig. 1*A*. YIp2-642ADR1-lacZ was constructed by inserting a 3-kb *Bam*HI fragment of *lacZ* from pMC1871 into the *Bgl* II site of pBC72, such that the open reading frame of the *ADR1* gene was fused at codon 642 in frame with *lacZ*.

Western Immunoblot Assay of ADR1. Protein extracts were made as described above, and protein concentrations were determined and verified by Coomassie blue staining of SDS gels. After electrophoresis of extracts through a 6.5% polyacrylamide/SDS gel and Western blotting onto a nitrocellulose sheet, the ADR1 protein was detected by using rabbit antisera to either synthetic ADR1 finger-region peptides ADR1a and ADR1c (18) or a TrpE–ADR1 fusion protein (unpublished data) as described (21).

ADR1 DNA Binding by Gel Retardation. Gel retardation assays were done essentially as described (20).

RESULTS

Overproduction and Identification of ADR1 in Yeast. To aid in the identification and characterization of ADR1, the wild-type ADR1 protein and the constitutive mutant ADR1-5^c were overproduced in yeast by fusing the genes to the strong constitutive *ADH1* promoter on the high-copy plasmids YEp1-ADR1 and YEp1-ADR1-5^c, respectively. The *ADR1* gene and an *ADR1–lacZ* derivative were also fused with the ADR1-activated glucose-repressible *ADH2* promoter to overproduce the proteins in an autocatalytically regulated manner from the yeast integrative plasmids YIp2-ADR1 and YIp2-642ADR1-lacZ (Fig. 1*A*). To measure the amount of ADR1 production, yeast transformed with these plasmids were analyzed by Northern blot hybridization (data not shown) and Western immunoblot procedures to detect ADR1 mRNA and protein, respectively.

Western immunoblots of protein extracts from derepressed yeast containing integrated YIp2-ADR1 or the ADR1–lacZ fusion plasmid showed protein bands having the expected mobilities for ADR1 (150 kDa) and 642ADR1-βgal (185 kDa), respectively, which were not detectable in extracts from the parent strain that lacked ADR1 (Fig. 1*B*, lanes 1–3). Western immunoblot analysis of extracts made from repressed or derepressed wild-type yeast cultures revealed equal amounts of ADR1 protein (Fig. 1*B*, lanes 4 and 5). When yeast were transformed with YEp1-ADR1, the band at 150 kDa increased about 20-fold in intensity during both repression and derepression, as expected when the strong constitutive ADH1 promoter drove transcription of the ADR1 gene on a plasmid (lanes 6 and 7). The ADR1 band at 150 kDa was greatly diminished when an excess amount of the trpE–ADR1 polypeptide antigen was used as a competitor (lanes 9 and 10) or when control extracts from an ADR1 deletion strain were analyzed (lane 8). These results indicated that intact ADR1 protein was equally abundant during repression, when it is unable to activate *ADH2* expression, and after derepression, when it is responsible for a >100-fold increase in *ADH2* expression.

Yeast ADR1 Binds UAS1 DNA. Gel-retardation experiments using cell extracts from yeast containing YEp1-ADR1 (or a control plasmid) showed one major ADR1-dependent complex and a minor ADR1-dependent complex of slower mobility (Fig. 2*A*, lanes 3 and 6). ADR1-dependent complex formation was dependent on UAS1 as expected (compare with lane 9; see probes in Fig. 2*B*).

In confirmation that the UAS region of the *ADH2* promoter is sufficient for ADR1 binding, a specific ADR1-dependent complex appeared when either UAS1–UAS2 or UAS1 alone was used as the radioactive DNA probe (Fig. 2*A*, lanes 10–14). Competition experiments showed that these ADR1-dependent complexes were specific in all cases (data not shown). These results are consistent with the DNA binding observed for an ADR1–β-galactosidase fusion protein produced in *Escherichia coli* (20).

DNA Binding Is Unaffected by ADR1 Inactivation. To test if *ADR1* inactivation by glucose repression involving cAPK is mediated by decreased DNA binding to *UAS1*, we studied DNA binding of ADR1 produced in glucose-repressed and -derepressed yeast or in cAPK mutants. ADR1-dependent bands were detected using wild-type yeast extracts (Fig. 3*B*, lanes 1 and 2). The binding activity of ADR1 was independent of the carbon source used to grow the yeast prior to preparation of the extracts. Western immunoblot analysis showed that the two extracts had similar low levels of ADR1 protein (Fig. 3*C*, lanes 1 and 2). The extract prepared from repressed wild-type yeast had no detectable ADHII activity, as expected (Fig. 3*A*, lane 1), whereas the extract prepared from derepressed wild-type yeast had ADHII activity at ≈700 milliunits/mg (Fig. 3*A*, lane 2). This indicated ≈30% of maximal derepression at the time the cells were collected.

A more sensitive way to measure ADR1-dependent DNA binding was provided by strains overproducing ADR1. Extracts were prepared from four congenic strains after repressed or derepressed growth. The effect of ADR1 over-

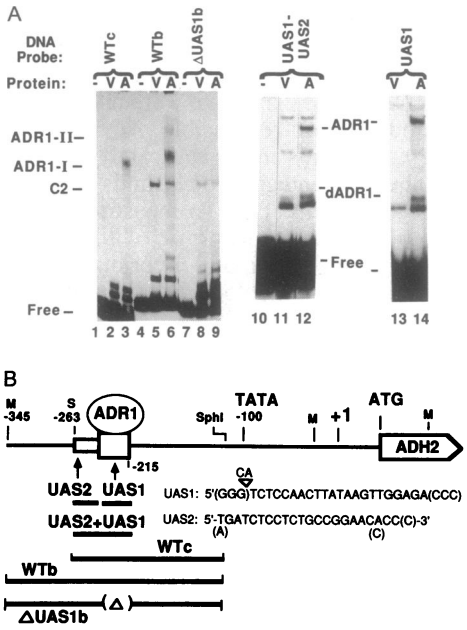

FIG. 2. ADR1 protein binding with UAS1 DNA. (*A*) Gel-retardation assays contained no protein (lanes −) or 15 μg of yeast protein extracts from strain 521-6-Δ1 transformed with YEp1-ADR1 (lanes A) or the vector pYcDE2 (lanes V) grown for 10 hr in derepressing conditions. Protein extracts were combined at 23°C with the indicated radioactive DNA probes (1–5 ng; see *B*) shown in the *ADH2* promoter map (see *B*) prior to an 8-min incubation, gel electrophoresis, and autoradiography. Mobilities of ADR1-dependent complexes, degraded ADR1 complexes (dADR1), another protein complex (C2), and free DNA are shown. (*B*) DNA fragments of the *ADH2* promoter used as protein–DNA binding probes WTc, WTb, and ΔUAS1b (WTb lacking UAS1) are shown below the map (20), including locations of sites for *Mbo* II (M), *Sau*3A (S), and *Sph* I relative to the transcription start site (+1). The sequences of DNA oligomers for UAS1 (a 22-bp inverted repeat extending from bp −237 to bp −216, flanked by GGG---CCC) and UAS2 [extending from bp −265 to bp −244 (24)] are shown. The sequence of the UAS2+UAS1 oligomer includes these two sequences plus the changes indicated (adenosine substituted for thymidine at the 5′ end, deletion of adenosine from bp −246 of UAS2, and proper inclusion of CA at bp −238 upstream of UAS1).

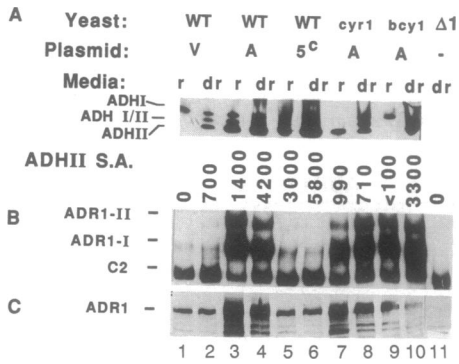

FIG. 3. *ADH2* gene activation and DNA binding of ADR1 from wild-type yeast and from yeast mutants of the cAPK pathway. (*A*) ADH activity was detected by native gel electrophoresis of 100 μg of yeast protein extracts from repressed (lanes r) or derepressed (lanes dr) cell cultures (described in *B*), followed by chromogenic staining for ADH (13). ADHII specific activity was determined by measuring total ADH activity using, separately, three substrates: ethanol, butanol, and isopropanol. The three ADH isozymes in wild-type yeast have different activities from one another on each of these three substrates. This difference allows the specific activity of ADHII to be estimated in a crude extract containing a mixture of the three isozymes. (*B*) Gel-retardation assays (see Fig. 2) were done using radioactive DNA probe WTc with 50 μg of protein extract from strain 521-6-Δ1 (lane Δ1), the wild-type yeast strain CMY-215 (lanes WT), and the cAPK pathway mutants KD100-13 (lanes bcy1) or CM1-2 (lanes cyr1) containing plasmids pYcDE2 (lanes V), YEp1-ADR1 (lanes A), or YEp1-ADR1-5c (lanes 5c) grown in repressing (lanes r) or derepressing (lanes dr) medium as indicated. (*C*) Western blots of ADR1 in various extracts performed after electrophoresis of 100 μg of protein in an SDS/polyacrylamide gel. Other experiments confirmed that the levels of ADR1 overexpressed from both *bcy1* and wild-type yeast were similar to each other in both repressed and derepressed cells containing YEp1-ADR1.

production on *ADH2* expression in wild-type yeast is seen in Fig. 3*A*, lanes 1 and 2 (control plasmid) versus lanes 3 and 4 (YEp1-ADR1). As shown by the pattern of ADH isozymes and the ADHII activity measurements, ADR1 overproduction led to constitutive *ADH2* expression and 6-fold enhanced derepression. Overproduction of a mutant form of ADR1, encoded by the *ADR1-5c* allele, led to even higher levels of constitutive expression and more extensive derepression (Fig. 3*A*, lanes 5 and 6). Overproduction of ADR1 in a *cyr1-2* mutant that has impaired cAPK activities also led to constitutive *ADH2* expression but less-extensive derepression than in the congenic wild-type strain (Fig. 3*A*, lanes 7 and 8). Overproduction of ADR1 in a *bcy1* mutant that has high unregulated cAPK activity did not lead to constitutive *ADH2* expression but did allow *ADH2* derepression comparable to that observed in the derepressed congenic wild-type strain (Fig. 3*A*, lanes 9 and 10). In contrast, we found that, in this *bcy1* strain containing only a single-copy *ADR1* gene, no derepression was observed (data not shown). This indicates that high-level ADR1 expression was able to overcome the *bcy1* defect but only during derepression. The ability of *bcy1* yeast to derepress *ADH2* expression was also dependent on the presence of lactate in the growth medium. Lactate allowed the *bcy1* strain to grow, albeit poorly, in the absence of a fermentable carbon source. In the presence of glycerol, ethanol, or both, neither cell growth (5, 6) nor *ADH2* derepression was observed.

The DNA binding activity of ADR1 in these extracts was assessed by gel-retardation assays (Fig. 3*B*) and the amount of ADR1 protein in each extract was estimated by Western blot analysis (Fig. 3*C*). There was a good correlation between the amount of ADR1-dependent complex formed and the amount of ADR1 protein present in the extract. In contrast there was a lack of correlation between the DNA binding activity of ADR1 and *ADH2* expression. For example, comparing samples prepared from wild-type yeast containing a single-copy *ADR1* gene that was repressed or derepressed (lanes 1 and 2, respectively), there was comparable DNA binding activity but 100-fold more ADHII activity after derepression. YEp1-ADR1-5c extracts had ADR1 antigen levels ≈2-fold higher than the control and had DNA binding activity that was commensurate with this level of ADR1 protein but contained much higher levels of ADHII (lanes 5 and 6), suggesting that ADR1-5c protein has normal DNA binding activity but is a particularly potent activator of transcription.

DNA binding activity of "active" versus "inactive" ADR1 was compared in several different ways. ADR1 from *bcy1* cells overexpressing ADR1 but not expressing ADH2 during repression (Fig. 3*A*, lane 9) and from wild-type derepressed cells overexpressing ADR1 (Fig. 3*A*, lane 4) was compared by varying the amount of ADR1-containing extract in the gel-retardation assay (Fig. 4*A*). ADR1 binding activity in the two extracts was indistinguishable at each concentration tested. These two extracts as well as extracts from repressed or derepressed wild-type cells not overexpressing ADR1 were also compared by using a constant amount of extract and an increasing amount of DNA. Fig. 4*B* shows that the amount of ADR1–DNA complex formed was again indistin-

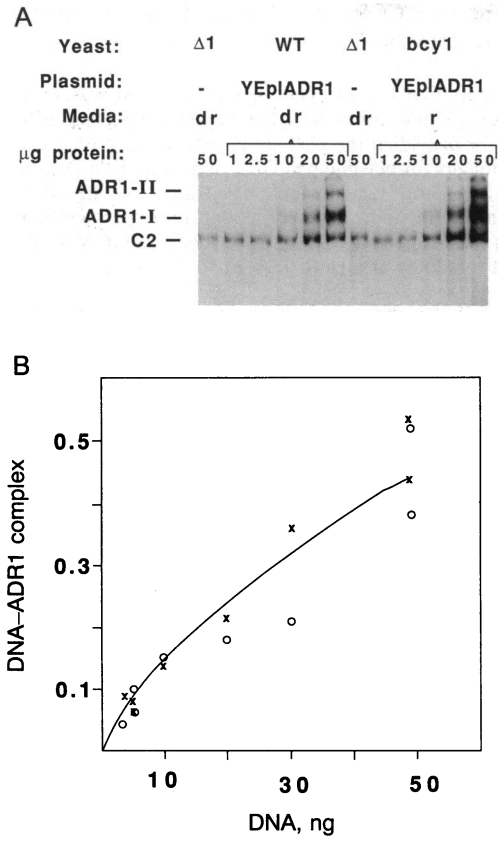

FIG. 4. DNA binding by ADR1 from yeast expressing or not expressing *ADH2*. (*A*) Gel retardation assays were performed using WTc probe DNA and 50 μg of total protein per reaction mixture. The extract protein was derived from strain 521-6-Δadr1 (lanes Δ1). CMY215:YEp1-ADR1 (lanes WT), or KD100-13:YEp1-ADR1 (lanes bcy1), grown in repressing (lanes r) or derepressing (lanes dr) medium as indicated. Each assay contained a constant amount of total extract protein (50 μg), of which an increasing amount was derived from the strain overexpressing ADR1. Thus, the non-ADR1-dependent complexes (C2) are relatively constant. (*B*) Gel-retardation assays were performed with a constant amount of extract protein (50 μg) from repressed (○) or derepressed (×) wild-type yeast (CMY215) lacking a plasmid containing *ADR1*. See Fig. 3 for a comparison of the ADHII activity and ADR1 content of these extracts. The UAS1–UAS2 DNA probe concentration was varied from ≈0.2 ng to 10 ng. After electrophoresis and autoradiography, the autoradiogram was scanned with a microdensitometer and the ADR1-dependent complexes were quantified by cutting out and weighing the tracing representing these bands on the gel. The ordinate represents these values in arbitrary units.

guishable using extracts that contained equal amounts of ADR1 either in an inactive (repressed) or in an active (derepressed) state.

The DNA binding by ADR1 in all of these extracts was competed identically by excess nonspecific DNA (data not shown). No difference in the rates of dissociation of active and inactive ADR1–DNA complexes was detected (data not shown). Thus, by several tests the DNA binding activity of ADR1 in extracts prepared from cells actively expressing *ADH2* or in which *ADH2* is completely repressed is unaffected.

These results suggest that ADR1, which is unable to activate transcription *in vivo*, is able to bind DNA *in vitro*. The major caveat to this conclusion is the possibility that the modification(s) that inactivate ADR1 *in vivo* are reversed during preparation of the extracts used for DNA binding. Since phosphorylation is the modification predicted to inactivate ADR1 (11), we tried to demonstrate that a phosphorylated form of ADR1 could bind to DNA. Extracts from glucose-grown cells overproducing ADR1 were treated with bovine cAPK plus ATP. Radioactive [γ-^{32}P]ATP incorporated into ADR1 could be detected by SDS gels and autoradiography (data not shown). This treatment did not, however, affect the DNA binding activity of ADR1 (Fig. 5*A*, lanes 1–4). In a second experiment, extracts were prepared from *bcy1*-repressed cells overproducing ADR1 and grown in the presence of inorganic [^{32}P]phosphate. Radioactive ADR1 was present in this extract as shown by immune precipitation in the presence and absence of competitor polypeptide (Fig. 5*B*, lanes 1 and 2). A preparative gel-shift experiment was performed in the presence or absence of nonradioactive probe DNA and slices of the gels corresponding to the ADR1-dependent DNA complex in a control lane were excised. Since the exact location of the ADR1–DNA complex in the preparative lanes might differ slightly from its position in the control lane, slices above and below that position were also excised, and proteins were electroeluted and analyzed.

ADR1 could be detected by Western analysis only from the gel in which unlabeled DNA probe was present during the

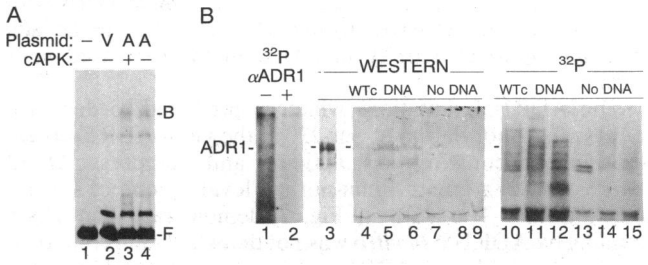

FIG. 5. Gel-mobility-shift analysis of phosphorylated ADR1 protein–DNA complexes. (*A*) Binding of ADR1 protein treated with cAPK to UAS1. DNA-binding reaction mixtures contained radioactive probe WTc and no protein (lane −) or yeast extracts made from 20B12 containing plasmids pYCDE2 (lane V) or YEp1-ADR1 (lane A) grown selectively in glucose medium. Phosphorylation of ADR1 protein extracts by bovine cAPK (lane +) was as described by Scott *et al.* (29). Lanes − contain samples without cAPK. Only probe DNA was loaded in lane 1. The position of free DNA probe (F) and specific ADR1 protein–DNA complexes (B) are indicated. The fast-migrating ADR1-dependent bands in lanes 3 and 4 probably represent proteolyzed fragments of ADR1 containing the DNA-binding domain. (*B*) ^{32}P-labeled ADR1 is present in a protein–DNA complex. Strain KD100-13 (*bcy1*) containing YEp1-ADR1 grown in glucose medium was labeled with [^{32}P]orthophosphate and portions of the extract were immunoprecipitated either in the absence (lane 1) or in the presence (lane 2) of specific ADR1 competitor polypeptide and analyzed by SDS gel electrophoresis and autoradiography. The notation ADR1 indicates the position of full-length ADR1 based on Western blot analysis with ADR1 antiserum of other lanes containing ADR1 (lane 3). ^{32}P-labeled ADR1 from protein–DNA complexes was detected by (*i*) Western immunoblot analysis with anti-ADR1 antiserum of proteins extracted from the appropriate region of a preparative gel retardation assay with 1 μg of nonradioactive WTc DNA (lanes 4–6) or no DNA (lanes 7–9) and by (*ii*) autoradiography (lanes 10–12 and 13–15). This protein extract (200 μg) was incubated in DNA binding buffer in the presence of 1 μg of nonradioactive WTc probe DNA or in the absence of WTc DNA. After electrophoresis a slice of polyacrylamide gel expected to contain a specific ADR1–DNA complex was excised based on the position of a radioactive WTc probe bound to nonradioactive ADR1 and electrophoresed on the same gel. Slices just above and just below this position were also excised. Protein was electroeluted, acetone-precipitated in the presence of carrier bovine serum albumin, dissolved in SDS sample buffer, and electrophoresed on a 6% polyacrylamide/SDS gel (lanes 4–6 and 10–12). Samples from the control gel (no WTc probe DNA added) were treated similarly (lanes 7–9 and 13–15). After Western blot analysis with anti-ADR1 antiserum and development with alkaline phosphatase reagents (lanes 4–9), the dried nitrocellulose sheet was exposed to film for autoradiography (lanes 10–15). Tick marks indicate the bands that are aligned with ADR1 in control lane 3.

binding reaction (Fig. 5*B*, lanes 4–6 versus lanes 7–9), indicating a DNA-dependent mobility shift of ADR1. Radioactive protein that migrated with a mobility corresponding to ADR1 was present only in the sample prepared from this gel (Fig. 5*B*, lanes 10–12 versus lanes 13–15). In addition, when an ADR1–β-galactosidase fusion protein produced in *E. coli* was phosphorylated *in vitro* by the catalytic subunit of bovine cAPK in the presence of [γ-^{32}P]ATP, radioactive fusion protein was shown to bind to a nonradioactive *ADH2* promoter probe (data not shown). Thus, by several criteria, phosphorylation of ADR1 does not prevent its binding to DNA.

DISCUSSION

ADR1 was identified as a 150-kDa protein that binds to UAS1, a 22-bp palindrome that is necessary and sufficient for ADR1-dependent activation of transcription (22, 23). Derepression of yeast in glucose-free medium had no detectable effect upon the integrity or the amount of ADR1 protein present or upon DNA binding of ADR1, although it activated ADR1-dependent gene expression of *ADH2* >100-fold. ADR1 from glucose-repressed *bcy1* yeast bound DNA with an affinity similar to ADR1 from *cyr1-2* cells, despite the lack of transcriptional activity of ADR1 in the repressed *bcy1* strain.

The ADR1-5^c mutation, which is predicted to decrease cAPK phosphorylation of Ser-230 in the yeast ADR1 protein (11), might constitutively activate and derepress *ADH2* expression to a greater-than-normal level by one of several mechanisms. The data in Fig. 3 demonstrate that DNA binding by ADR1-5^c *in vitro* was not detectably different than binding by wild-type ADR1, yet it activated *ADH2* expression significantly better. Thus, ADR1-5^c is a more potent activator of transcription. If ADR1 always bound DNA and some ADR1 is always in a phosphorylated inactive form, then a mutant that could not be phosphorylated would have maximal activity.

There are several indications that phosphorylation by way of the cAPK pathway inactivates ADR1 (ref. 11; K. Dombek, personal communication). We were unable to demonstrate that phosphorylation of ADR1 had an effect on its ability to bind DNA *in vitro*. Moreover, inactive ADR1 phosphorylated *in vivo* in a *bcy1* strain was able to bind normally to UAS1 in the *ADH2* promoter. Thus it seems probable that if phosphorylation does indeed inactivate ADR1, then it does not do so by preventing ADR1 from binding to DNA. One possibility is that ADR1 protein, which is constitutively expressed in many but not all yeast strains (15), remains bound to UAS1 in the nucleus (17). During glucose repression, cAMP-dependent phosphorylation of ADR1 may inhibit its ability to interact with general transcription factors or RNA polymerase II.

Current evidence is consistent with cAPK phosphorylation inactivating the ability of ADR1 to stimulate *ADH2* expression during growth in glucose, but the nature of the change to an active state during derepression is unclear. Derepression of *ADH2* requires several factors besides the ADR1 protein, including pleiotropic factors ADR6 (30), a protein kinase, CCR1 (SNF1) (31, 32), and CCR4, which is negatively regulated by CRE1 and CRE2 (33). Since *ADH2* can be derepressed after inhibition of new protein synthesis with cycloheximide (K. Dombek, personal communication), these factors must be present in repressed yeast. Enzymes such as a protein-serine phosphatase or a second type of protein kinase (perhaps CCR1) could mediate the activation of ADR1 during derepression.

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