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Glucose Repression of the Yeast ADH2 Gene Occurs through Multiple Mechanisms, Including Control of the Protein Synthesis of Its Transcriptional Activator, ADR1†

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The rate of ADH2 transcription increases dramatically when Saccharomyces cerevisiae cells are shifted from glucose to ethanol growth conditions. Since ADH2 expression under glucose growth conditions is strictly dependent on the dosage of the transcriptional activator ADR1, we investigated the possibility that regulation of the rate of ADR1 protein synthesis plays a role in controlling ADR1 activation of ADH2 transcription. We found that the rate of ADR1 protein synthesis increased 10- to 16-fold within 40 to 60 min after glucose depletion, coterminal with initiation of ADH2 transcription. Changes in ADR1 mRNA levels contributed only a twofold effect on ADR1 protein synthetic differences. The 516-nt untranslated ADR1 mRNA leader sequence was found to have no involvement in regulating the rate of ADR1 protein synthesis. In contrast, sequences internal to ADR1 coding region were determined to be necessary for controlling ADR1 translation. The ADR1c mutations which enhance ADR1 activity under glucose growth conditions did not affect ADR1 protein translation. ADR1 was also shown to be multiply phosphorylated in vivo under both ethanol and glucose growth conditions. Our results indicate that derepression of ADH2 occurs through multiple mechanisms involving the ADR1 regulatory protein.

In the yeast Saccharomyces cerevisiae, glucose represses the transcription of numerous genes, including those required for ethanol and alternate sugar metabolism, the glyoxylate shunt, the tricarboxylic acid cycle, respiration, gluconeogenesis, and mitochondrial function. Several regulatory genes which, when mutated, either allow glucose-insensitive transcription or inhibit derepression upon glucose removal have been identified (34). The mechanisms by which glucose repression occurs, however, remain largely obscure. We have previously implicated the glucose-induced adenylyl cyclase signaling system in the inhibition of alcohol dehydrogenase II (ADH II; ADH2 gene) expression (7, 13, 16). This mechanism, though, would account for only a small portion of the total glucose repression of ADH2 (7, 14). We show in the present study that glucose-dependent control of ADR1 protein synthesis contributes significantly to the overall glucose regulation of ADH2 expression.

The ADR1 regulatory protein is a transcriptional activator of the ADH2 gene (14, 33) and is required for the dramatic 500-fold increase in ADH II activity, which is initiated upon depletion or removal of glucose from the growth medium (8). Activation of ADH2 transcription requires the binding of ADR1 protein to a 22-bp dyad activation sequence in the noncoding region of the ADH2 gene (35). Evidence indicates that such binding occurs through two zinc fingers located in the N-terminal region of ADR1 (35) and that the ability of ADR1 to bind ADH2 appears to be carbon source independent (31). In addition to its control of ADH2, ADR1 is required for transcription of genes involved in peroxisome function (28) and for undefined factors required for nonfermentative growth (2).

Glucose regulation of ADR1 function appears to occur principally at the posttranscriptional level since ADRI mRNA levels do not differ significantly between glucose- and ethanol-grown cells (4, 12, 17). Dominant mutations in ADR1 (designated ADR1c), which allow glucose-insensitive ADH2 transcription (14), have been identified as point mutations causing single amino acid substitutions between amino acids 227 and 239 of ADR1 (7, 16, 17). While it was originally postulated that these mutations affected the cyclic AMP-dependent protein kinase phosphorylation site at ser-230 of ADR1 (7, 17), more recent evidence indicates that this protein kinase inhibits ADR1 function by a mechanism that is independent of effects on ser-230 (16). How ADR1c mutations activate ADR1 under glucose growth conditions remains unclear. It is apparent, however, that additional mechanisms controlling ADR1 function must be operative, since ADR1c-containing strains remain partly subject to the effects of glucose repression (10, 14).

We have reported previously that ADH2 expression under glucose growth conditions increased linearly in response to increased ADR1 dosage (12). This observation raises the possibility that changes in the rate of ADR1 protein synthesis may play a role in the control of ADR1 activation of ADH2 transcription. We report here that the rate of ADR1 protein synthesis increased dramatically within the first 40 min of shifting cells from glucose to ethanol growth medium.

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MATERIALS AND METHODS

Yeast strains. All yeast strains used in this study are isogenic to strain 500-16, MAL \textit{a} adh1-11 adh3 ura1 his4 trp1, and have been described previously (2, 12).

Northern analysis. Cells were grown overnight at 30°C in YEP medium consisting of 2% Bacto Peptone, 1% yeast extract, 20 mg of adenine and uracil per liter, and either 8% glucose or 3% ethanol. Cultures were inoculated from YEP plates containing 2% agar and either 8% (YD plate) or 2% (YD plate) glucose.

Total yeast RNA was extracted as previously described (15). Northern (RNA) analysis was conducted according to the manufacturer's specifications as described in the New England Nuclear GeneScreen Instruction Manual. The \textit{ADR1} hybridization probe, a 3.4-kb HindIII fragment from plasmid YRp7-ADR1-23A (17), was radiolabeled by using a random priming kit (Boehringer-Mannheim). \textit{ADR1} RNA levels were quantitated by densitometric analysis with an EC610 densitometer and normalized to the amount of rRNA present when comparing samples grown under glucose and ethanol growth conditions (15, 18). rRNA has been found to be a useful standard for comparing \textit{ADR1} mRNA extracted from cells harvested from the different growth conditions (3, 12, 15, 17). The wild-type strain used in this study, which lacks ADH I activity, grows at the same rate in medium containing glucose as in medium containing ethanol, obviating a carbon source variation in RNA levels (23). Moreover, other experiments have indicated that normalization to \textit{CCR4} mRNA levels gives results in agreement with those obtained by using rRNA as a standard. Normalization to the level of \textit{URA3} RNA was used as another standard when comparing samples from cells grown under the same growth conditions, yielding results in agreement with those obtained from the use of rRNA as standard.

Plasmid construction. Strain WC1 was derived from 500-16 by site-directed integration of the G-\textit{ADR1} allele at the \textit{adr1-1} locus. Plasmid G-ADR1 was constructed from plasmid JS119, which contains the complete \textit{ADR1} gene under the control of the \textit{G3PDH} promoter (7), by ligating a \textit{BamHI} fragment containing the \textit{G3PDH} promoter and the first 3,200 bp of \textit{ADR1} into YRp7 at its \textit{BamHI} site. The resultant G-ADR1 plasmid was cut in the interior of the \textit{ADR1} gene with \textit{SsrI} (at bp 1715) prior to transformation of strain 500-16.

In vivo labelling of yeast proteins with \textit{35S}-amino acids. In vivo labelling of yeast cell proteins with \textit{35S}-amino acids was conducted by the method essentially as described by Reid (27). Cells labelled under glucose growth conditions were prepared by inoculation from a YD8 plate into liquid growth medium made with sulfate-free salts and containing 8% glucose. Cells labelled under ethanol growth conditions were prepared by inoculation from a YD plate into the same sulfur-deficient medium but with the substitution of 3% ethanol for the glucose. Following overnight growth (17 to 20 h) at 30°C in a shaker-incubator, the yeast cells were harvested by centrifugation. Cells were resuspended in fresh media of the same composition used for overnight growth but containing an added specified amount (typically 200 to 250 \textmu Ci/ml of culture) of Tran \textit{[35S]} label (ICN Corp.). Tran \textit{[35S]} label contains ca. 70% \textit{[35S]}Met and ca. 15% \textit{[35S]}Cys. Cultures were returned to the shaker-incubator for labelling during further growth at 30°C until harvesting or further treatment. The time allowed for pulse-labelling varied from 15 min to 2 h, as indicated in the text. Control experiments demonstrated incorporation of \textit{35S} label into total yeast proteins to be linear as a function of time and the rates of incorporation to be nearly identical under glucose and ethanol growth conditions. The levels of ADH II activity were determined (11) for each strain grown under the conditions described above to be unchanged from the activity levels determined for cells grown in the typical YEP growth medium.

In vivo labelling of yeast proteins with \textit{32P} phosphate and \textit{32P} thio phosphate. In vivo labelling of yeast cell proteins was accomplished by incubating cells, pregrown in YEP medium and then washed once with low-phosphate medium (UMD medium [5] containing 0.3 mg of KH\textsubscript{2}PO\textsubscript{4} per ml and the appropriate carbon source (3% [vol/vol] ethanol or 8% [wt/vol] glucose), at 30°C with shaking in low-phosphate medium containing either 3% (vol/vol) ethanol or 8% (wt/vol) glucose and 0.5 mCi of \textit{32P} thio phosphate (New England Nuclear) or \textit{32P} orthophosphate (New England Nuclear) per ml of culture. In control experiments, \textit{ADR1} was phospho- labeled in vivo to the same extent by using either \textit{32P} phosphate or \textit{32P} thio phosphate. Incubation times varied from 15 min to 3 h according to each experiment.

Pulse-chase and growth-shift experiments. For the pulse-chase experiments, total yeast proteins were radiolabeled for 2 h with \textit{35S}-amino acids as described above. The chase was accomplished by adding sufficient unlabelled methionine to give a final concentration of 2 mM in the culture. Extracts were prepared from portions of the culture harvested at selected times following addition of the unlabelled methionine. In experiments in which cells were shifted from glucose- to ethanol-containing medium, cells were rapidly washed twice with distilled water before being resuspended in growth medium containing 3% ethanol. Shifted cultures were pulsed as described above for 20 min with \textit{35S}-amino acids at the times indicated in Fig. 9.

Preparation of yeast cell extracts. Pulse-labelled cells, harvested after centrifugation and removal of the labelling medium, were suspended in 0.4 ml of cold lysis buffer (1.0 mM EDTA, 0.50% [wt/vol] sodium dodecyl sulfate [SDS], 0.01 mg of decapeptide per ml [Sigma], 0.01 mg of peptatin per ml [Sigma], 1 mM phenylmethylsulfonyl fluoride [PMSF] [Sigma], 1% aprotinin [Sigma], 1 mM dithiothreitol, 10 mM Tris-Cl, pH 7.4) and vortexed in the cold for 45 s in glass tubes containing sterile glass beads to accomplish cell lysis. Cell extracts were removed from the glass beads into 1.5-ml microfuge tubes, using an additional 0.6 ml of lysis buffer to rinse the beads and to assure a quantitative transfer of the extract. The resulting extracts (ca. 1.0- to 1.1-ml final volumes) were boiled in capped microfuge tubes for 5 min, cooled to room temperature, and centrifuged for 10 min to remove cell debris and other insolubles. Nine hundred microliters of each supernatant (the clarified whole yeast cell extract) was removed to fresh microfuge tubes and mixed with 100 \mu l of 10% (vol/vol) Nonidet P-40 (Sigma). Fresh PMSF and aprotinin were added to concentrations of 1 mM and 1%, respectively, before the mixture was frozen at -20°C.

Immunoprecipitation of \textit{ADR1} from SDS-denatured yeast cell extracts. \textit{ADR1} protein labelled in vivo with \textit{35S}-amino acids was immunoprecipitated from SDS-denatured yeast cell extracts (prepared as described above) by using the protein A-agarose method (22) with slight modifications. Nonidet P-40-neutralized SDS-denatured extracts were pre-cleared by being mixed end over end for 15 min at 4°C with 0.4-ml (packed volume) of protein A-agarose-nonimmune IgG (prepared by mixing equal volumes of protein A-agarose [Boehringer Mannheim] and nonimmune serum end over end.
for 30 min at 4°C followed by being washed with 3 15-ml volumes of Triton wash buffer [0.05% (vol/vol) Triton X-100, 0.14 M NaCl, 1 mM EDTA, 20 mM potassium phosphate, pH 7.0)]. Following a brief centrifugation to pellet the protein A-agarose-nonimmune IgG and nonspecifically bound yeast proteins, the precleared extracts were removed to fresh microfuge tubes. Portions (5 μl) of each precleared extract were transferred in triplicate to separate Whatman 3MM filter paper disks. Each disk was treated under suction in a Millipore rinsing manifold with 3 15-ml volumes of ice-cold 1% (wt/vol) trichloroacetic acid (TCA) and 1 15-ml volume of ice-cold 95% ethanol. Disks were air dried and transferred to scintillation vials containing 5 ml of Aquasol before the radioactivity was counted in a Beckman liquid scintillation counter.

Appropriate volumes of precleared yeast extracts (enough to give 5.0 × 10⁶ TCA-precipitable cpm, unless otherwise indicated) were incubated for 2 h on ice with 10 μl of immune sera, preimmune sera, or immune sera pretreated for 10 min with an excess of the antigenic ADR1 peptide. In some experiments, unlabelled yeast extract lacking the ADR1 protein was added to the incubations to reduce nonspecific binding of labelled yeast proteins to the antibodies and protein A-agarose. Immune complexes were precipitated by the addition of 50 μl of a 50% (vol/vol) suspension of protein A-agarose in Triton wash buffer and then mixed end over end for 20 min at 4°C. After centrifugation for 30 s in a microfuge, the pellets were washed five times with 1.5-ml volumes of ice-cold RIPA buffer (10 mM sodium phosphate [pH 7.0] containing 175 mM NaCl, 1% [vol/vol] Nonidet P-40, 0.1% [wt/vol] SDS, and 1% sodium deoxycholate). The washed pellets were resuspended in 0.1 ml of SDS sample buffer (0.1 M Tris [pH 6.8], 10% [vol/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol, 1% [wt/vol] SDS) and heated in a boiling water bath for 3 min. The immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by fluorography. The intensities and patterns of bands corresponding to nonspecifically precipitated proteins were found to vary from one experiment to the next. In contrast, specific precipitations of ADR1, as determined by comparison to immunoprecipitates preincubated with excess antigenic peptide, were found to be highly reproducible. Among the likely possible sources producing variable background levels of non-ADR1 proteins were (i) different batches of protein A-agarose having different capacities for nonspecific binding of yeast proteins, (ii) the presence of different levels of nonlabelled yeast proteins competing for nonspecific binding sites, (iii) variable effectiveness of the preclearing step, (iv) variable effectiveness of the immunoprecipitated-pellet wash step, and (v) the use of different preparations of antiserum. It was observed, however, that for immunoprecipitations conducted on a given day with different yeast extracts, differences in background between immunoprecipitations were minimal (e.g., see Figs. 5 and 7-9).

Western blot (immunoblot) analysis of ADR1 protein in whole yeast cell extracts. Polyacrylamide gels were soaked in transfer buffer (20% methanol, 0.025 M Tris [pH 8.3], 0.192 M glycine) for 20 min and then blotted to Immobilon transfer membrane at 60 V for 2 h. The membrane was subsequently soaked in 100 ml of Blotto (50 mM Tris [pH 8.0], 2 mM CaCl₂, 80 mM NaCl, 20% methanol, 0.2% NP40) for 1 h at room temperature, washed three times for 5 min each in phosphate-buffered saline (PBS)-TWEEN (0.14 M NaCl, 1 M KCl, 15 mM KH₂PO₄, 0.15 mM K₂HPO₄, 0.1% Tween 20), and then incubated for an additional hour in Blotto with 30 μl of anti-ADR1 208-231 peptide antibody. The membrane was rewashed three times in PBS-Tween, for 5 min each wash, and reincubated for 1 h at room temperature in Blotto with 60 μl of horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham). The membrane was washed with PBS-Tween three times and then with PBS for 5 min each time. The blot was developed with 32 mg of diaminobenzidine in 100 ml of PBS with 60 μl 30% H₂O₂ as a substrate.

Peptide synthesis and antibody production. ADR1 peptides comprising amino acid residues 2 to 21 and 208 to 231 were synthesized by the method of Merrifield as previously described (29). Conjugation of peptides to bovine serum albumin (BSA) and production of antibodies have been described previously (24, 25, 32). Preimmune and immune sera were used without further purification. Anti-ADR1-208-231 peptide antibody immunoprecipitation of ADR1 polypeptide was found to be unaffected by phosphorylation of Ser-230 or mutation of the Arg-228 to lysine (ADR1-5'-allele) or Ser-230 to leucine (ADR1-7'-allele) (data not shown).

Alkaline phosphatase treatment of ADR1. Treatment with alkaline phosphatase of immunoprecipitated radiolabeled ADR1 was accomplished by the method essentially as described by Barber and Verma (1). Radiolabeled ADR1 was liberated from the washed protein A-agarose immunoprecipitates by incubation for 2 min in a boiling-water bath following the addition of 100 μl of 50 mM Tris (pH 8.0) containing 2 mM PMSF, 5 mM dithiothreitol, and 0.3% (wt/vol) SDS. Following a brief spin (5 to 7 s) in the microfuge, the supernatant containing the labelled ADR1 was transferred to fresh microfuge tubes and adjusted to contain 1% (vol/vol) Nonidet P-40, 1% (wt/vol) sodium deoxycholate (Sigma) and 150 mM NaCl. Neutralized ADR1 extracts were treated with 25 U of unwashed-calf alkaline phosphatase (Sigma) at 37°C. Reactions were terminated by the addition of SDS sample buffer and incubation for 3 min in a boiling-water bath. The resulting samples were analyzed by using the SDS-PAGE and fluorography system described above.

RESULTS

ADR1 protein identification. The ADR1 protein was immunoprecipitated from SDS-denatured extracts of yeast cells labelled in vivo with ³⁵S-amino acids. ADR1, immunoprecipitated with antibodies directed against a synthetic peptide corresponding to amino acid residues 208 to 231 of the ADR1 protein, migrated on SDS-polyacrylamide gels as a single species corresponding to a polypeptide of ca. 152 kDa (Fig. 1, lane 2). The identification of the polypeptide migrating at 152 kDa as ADR1 is supported by the following results: (i) the actual calculated molecular mass of unmodified ADR1 is 150,185 Da (20), (ii) the 152-kDa polypeptide did not immunoprecipitate from incubations in which preimmune sera were substituted for immune sera (Fig. 1, lane 1), (iii) the 152-kDa polypeptide did not immunoprecipitate from incubations containing immune sera preincubated with the antigenic peptide (Fig. 1, lane 3), (iv) the 152-kDa band was not detected in immunoprecipitates of cell extracts of a strain deficient in ADR1 expression (nonsense mutation in the 11th codon of the adr1-1 gene) [3] (Fig. 1, lane 4 (adr1-1) compared with lane 6 [multicopy ADR1]), (v) the intensity of the 152-kDa band increased in proportion to the number of integrated ADR1 gene copies contained in strains overexpressing the ADR1 gene (see Fig. 3 and 5), and (vi) the 152-kDa species was not detected in strains producing truncated versions of ADR1 protein, in which shorter versions of ADR1 were detected (e.g., see Fig. 10, lane 3). We have
obtained these results by using antisera raised against a peptide corresponding to amino acid residues 208 to 231 of ADR1 (Fig. 1 to 4) or a second synthetic peptide corresponding to residues 2 to 21 of the ADR1 protein (see Fig. 3 to 5 and 7 to 10). Taken together, our results provide conclusive evidence that the identity of the polypeptide migrating at 152 kDa in lane 2 of Fig. 1 is the ADR1 protein.

**ADR1 protein is multiply phosphorylated in both glucose- and ethanol-grown cells.** We observed during the course of the above immunoprecipitation experiments that the ADR1 signal broadened in the direction of higher-molecular-weight species as a function of the length of the labelling time (compare lanes 1 [15 min] and 2 [45 min] of Fig. 2). Such broadening suggests the likelihood that newly synthesized ADR1 protein is subject to rapid posttranslational modification. Thus, to ensure our accuracy in the identification and quantitation of all forms of newly synthesized ADR1 protein, we sought to identify the mechanism responsible for creating the observed ADR1 signal pattern.

Although direct biochemical evidence that ADR1 occurs in vivo as a phosphoprotein has not been reported, phosphorylation appeared to be a likely mechanism responsible for the observed broadening of the ADR1 signal. To investigate this hypothesis, we applied the ADR1 immunoprecipitation protocol described above to determine the in vivo phosphorylation state of ADR1 under glucose and ethanol growth conditions. ADR1 is shown in lane 4 of Fig. 2 after its immunoprecipitation from yeast labelled in vivo with [35S]thiophosphate under ethanol growth conditions. Phosphorlabelled ADR1 did not precipitate with preimmune sera (lane 3) or with immune sera that were preincubated with ADR1 peptide (lane 5). ADR1 was also shown to be a phosphoprotein under glucose growth conditions (data not shown; see also Fig. 2, lanes 6 to 8 and 12, and Fig. 4).

We observed phosphorlabelled ADR1 to be immunoprecipitated specifically by two different antisera raised against two different regions of the ADR1 protein (residues 2 to 21 and 208 to 231) (Fig. 4) and to comigrate on SDS-polyacrylamide gels with immunoprecipitated ADR1 labelled with [35S]methionine (Fig. 2, lane 6) (data not shown), confirming the identity of this species as phosphorylated ADR1. In addition, a strain lacking the full-length ADR1 protein but containing a truncated ADR1 gene (ADR1-262) lacks the phosphorylated species migrating at 152 kDa and instead contains peptide-blockable phosphorylated proteins that comigrate with 35S-amino-acid-labeled ADR1-262 (see Fig. 10, lanes 9 and 10 compared with lanes 5 and 6) (data not shown).

Alkaline phosphatase treatment of ADR1 immunoprecipitated from cells pulse-labelled for 2 h with 35S-amino acids caused the size of the ADR1 signal to be dramatically reduced and to resemble the thin band produced at 152 kDa by ADR1 from cells pulse-labelled for 15 min (Fig. 2, lanes 6 to 8 compared with lane 1). Treatment with alkaline phosphatase of ADR1 immunoprecipitated from cells labelled with [35S]thiophosphate caused the ADR1 signal to be reduced in breadth but not to disappear completely (lanes 9 to 11), suggesting that one or more of the phosphorylated residues may be resistant to the action of alkaline phosphatase. In contrast, incubation of ADR1 immunoprecipitated from cells labelled with [35S]thiophosphate in the

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**FIG. 1.** Identification of ADR1 by immunoprecipitation. Cultures (4 ml) of strain 411-1 (lanes 1 to 3) ([35S] copies of ADR1) were radiolabeled for 15 min with 210 μCi of [35S]-amino acids per ml, as described in Materials and Methods. Two hundred microliters of precleared extract was treated with 15 μl of preimmune sera (lane P), anti-ADR1-208-231 peptide antibody (lane E), or anti-ADR1-208-231 peptide antibody pretreated with excess ADR1 peptide (lane B). Equal volumes of the immunoprecipitates were separated on a 5% polyacrylamide gel. Cultures of strain 500-16 (adr1-1) (lanes 4 and 5) and strain 411-1 (lane 6) were radiolabeled as described for lanes 1 to 3 except 310 μCi of [35S]-amino acids per ml was used. To enhance detection of immunoprecipitated species, precleared extracts were first immunoprecipitated with anti-ADR1-208-231 antibody and then reimmunoprecipitated as described for lanes 2 and 3, respectively. Control experiments indicated that reimmunoprecipitation did not impair ADR1 detection. This figure is a composite of two different autoradiograms depicting the results of two different immunoprecipitation experiments (lanes 1 to 3 and lanes 4 to 6) and, as a result, shows variation in the patterns and intensities of nonspecific background bands.

**FIG. 2.** ADR1 is multiply phosphorylated. Lanes 1 and 2 compare the ADR1 signals in immunoprecipitates obtained by incubating anti-ADR1-208-231 peptide antiserum with extracts of yeast strain 411-1 (96 integrated ADR1 gene copies) labelled under glucose growth conditions for 15 and 45 min, respectively, with [35S]-amino acids. Lanes 3, 4, and 5 compare immunoprecipitates obtained by incubating the extract of yeast strain 411-1 labelled in vivo for 3 h with [35S]thiophosphate (350 μCi/ml) under ethanol growth conditions with preimmune serum, anti-ADR1-208-231 peptide antiserum, and anti-ADR1-208-231 peptide antiserum, that was blocked by preincubation with the antigenic peptide, respectively. The effect of in vitro alkaline phosphatase (AP) treatment on the breadth of the ADR1 signal from yeast strain 411-1 labelled in vivo either for 45 min with [35S]-amino acids under glucose growth conditions (lanes 6 to 8) or for 1 h with [35S]thiophosphate (0.4 mCi/ml) under ethanol growth conditions (lanes 9 to 11). ADR1 was either not treated with alkaline phosphatase (control) (lanes 6 and 9) or treated with alkaline phosphatase for 1 lane 7 and 10), 2 lane 11), or 3 (lane 8) h. Lanes 12, strain 411-1 labelled under glucose growth conditions as described for lane 9; 13, same as lane 12 except incubated for 2 h without alkaline phosphatase; 14, same as lane 13 except incubated with alkaline phosphatase.
FIG. 3. Glucose control of ADR1 protein synthesis. Yeast cells were labeled with $^{35}$S-amino acids and immunoprecipitated as described in the legend to Fig. 1 and Materials and Methods (lanes 1 to 14). Anti-ADR1-208-231 peptide antibody (lanes 1 to 6) and anti-ADR1-2-21 peptide antibody (lanes 7 to 14) were used. For glucose (lanes G) and ethanol (lanes Et) comparisons of the same strain, equivalent amounts of precleared extracts based on TCA-precipitable counts were incubated with 15 μl of immune sera (lane E), immune sera pretreated with excess ADR1 peptide (lane B), or preimmune sera (lane P). For lanes 1 to 6, precleared extracts from glucose-grown cultures (lanes G) contained twice the number of TCA-precipitable counts as that of ethanol-containing cultures (lanes Et). Black dots beside the ADR1 polypeptide indicate its position. The strains 411-40 (ADR1), 411-12 (ADR1 [16 copies of ADR1]), and WC1 (G-ADR1) which contains one copy of the ADR1 gene under the control of the weak GpdH promoter (Materials and Methods) are indicated above the lanes. Samples were separated on a 5% (lanes 1 to 6), 6% (lanes 15 and 16), or 7.5% (lane 7 to 14) polyacrylamide gel. Radioactive labelling was done for 15 (lanes 1 to 6), 90 (lanes 7 to 10) or 60 min (lanes 11 to 14). (Lanes 15 and 16) depict a Western blot in which equivalent amounts of total yeast extracts grown on YEP medium containing either 8% glucose (lanes G) or 3% ethanol (lanes Et) were blotted to Immobilon paper (Millipore) and treated with anti-ADR1-208-231 peptide antibody, as described in the Materials and Methods. This figure is a composite of several different autoradiograms as described in the legend to Fig. 1.

absence of alkaline phosphatase did not affect the breadth of the ADR1 signal (lane 12, no incubation; lane 13, no alkaline phosphatase treatment; and lane 14, alkaline phosphatase treatment). These results clearly define the forms of newly synthesized ADR1 and indicate that ADR1 is multiply phosphorylated in vivo under both glucose and ethanol growth conditions during or soon after its synthesis (within 45 min).

The rate of ADR1 protein synthesis is reduced in glucose-grown cells. We observed the amount of ADR1 immunoprecipitated from extracts of ethanol-grown cells to be consistently and significantly greater than the amount of ADR1 immunoprecipitated from extracts of cells grown on glucose, suggesting that ADR1 activity might be regulated through a carbon source-linked control of ADR1 protein synthesis. Figure 3 illustrates the results of an ADR1 immunoprecipitation experiment using SDS-denatured extracts of yeast strain 411-40 (containing a single copy of ADR1) pulse-labelled for 15 min with $^{35}$S-amino acids under glucose (lane 2) and ethanol (lane 5) growth conditions. As shown in the Fig. 3, we were able to detect ADR1 under ethanol growth conditions but not under glucose growth conditions. We obtained similar results by Western analysis of whole-cell (strain 411-40) extracts (Fig. 3, lanes 15 and 16, respectively), indicating that in addition to the rate of ADR1 protein synthesis, the steady-state level of ADR1 protein accumulated in the cell is also affected by the carbon source.

We subsequently analyzed yeast strains 411-26, 411-3, and 411-12 carrying 4, 9, and 16 copies, respectively, of the ADR1 gene integrated into the genome to estimate the minimum ADR1 dosage at which ADR1 protein could be detected, and ADR1 regulation in glucose-grown cells. ADR1 regulation in strains 411-26, 411-3, and 411-12 is identical to that of isogenic strain 411-40 carrying a single copy of the ADR1 gene except for commensurate dosage-dependent elevations in ADH II activity (2, 12). We were unable to detect ADR1 protein in strains 411-26 (four copies of ADR1) and 411-3 (nine copies of ADR1) grown under glucose growth conditions (data not shown). We obtained an ADR1 protein signal from each of these strains, however, that was clearly both visible and present in the expected relative proportions from extracts of ethanol-grown cells (data not shown). We determined that an ADR1 dosage of 16 (strain 411-12) yielded a reproducibly detectable ADR1 protein signal under glucose growth conditions that was roughly equivalent to that obtained under
ethanol growth conditions in the single copy strain (compare lanes 5 and 8 of Fig. 3). Densitometric analysis indicated that ADR1 protein accumulated in strain 411-12 (16 ADR1 copies) at about an 11-fold faster rate under ethanol growth conditions compared with glucose growth conditions. This result is in good agreement with our observation above that a dosage of 16 ADR1 gene copies is the approximate minimal dosage required for detection of ADR1 in glucose-grown cells.

We observed the same ADR1 abundance patterns, using two different antisera raised against peptides corresponding to two different regions (amino acids 208 to 231 and 2 to 21) of the ADR1 protein (compare lanes 1 to 6 with 7 to 18 of Fig. 3). This result provides evidence that the amounts of ADR1 protein observed in our immunoprecipitation experiments are accurate reflections of the quantities of newly synthesized ADR1 protein present in the cell extracts (and therefore, ADR1 protein synthetic rates) and are not attributable to carbon source-related bias in ADR1-antibody interaction. Such a bias might be envisioned to exist between the antisera raised against the ADR1-208-231 peptide (which contains a putative regulatable phosphorylation site at Ser-230) and the actual in vivo forms of the ADR1 protein. Previous experiments indicated, however, that the anti-ADR1-208-231 peptide antibody identifies ADR1 regardless of the phosphorylation state at Ser-230 or whether the serine site is altered to an amino acid that cannot be phosphorylated (7). In order to further confirm that the anti-ADR1-208-231 peptide antibody precipitates all phosphorylated ADR1 species, we compared the ability of the two antipeptide antibodies to precipitate ADR1 phosphorylated under glucose versus ethanol growth conditions. We observed no difference in either the intensity or banding pattern of ADR1 signals immunoprecipitated with each antiserum (Fig. 4, compare lanes a and d [anti-ADR1-208-231 antibody] with lanes b and e [anti-ADR1-2-21 antibody]).

ADR1p-type mutations do not affect ADR1 protein synthesis. Our observation that the rate of ADR1 protein synthesis is regulated by the carbon source suggests the possibility that the effect of ADR1p-type mutations might be to increase the rate of synthesis of the ADR1 protein. We therefore applied the labelling and immunoprecipitation techniques described above to investigate the potential effect of ADR1p-type mutations on ADR1 protein synthesis. Yeast strain 6-60 expresses a single copy of the ADR1-5p allele (R228K: contains a lysine at residue 228 instead of an arginine) and results in ADH II activities of ca. 300 mU/mg on glucose and ca. 5,000 mU/mg on ethanol (12). The isogenic strain 411-40 carrying the wild-type ADR1 gene by comparison yields ADH II activities of only 5 and 2,000 mU/mg on glucose and ethanol, respectively (12). Figure 5 demonstrates the disparity between the rates of ADR1 synthesis with the two carbon sources by comparing the amounts of the ADR1 protein immunoprecipitated from extracts of strain 6-60 following growth on glucose and on ethanol. As illustrated in lane 1 of Fig. 5, the immunoprecipitates corresponding to glucose-grown cells did not produce a discernible ADR1 signal. We repeated this experiment with strains 6-60 and 411-40 numerous occasions, each time attempting to adjust conditions (e.g., labelling time, amount of labelled extract in the immune reaction, gel conditions, and double immunoprecipitations) so as to maximize the likelihood of ADR1 protein detection. We were unable, however, to detect a band representing the ADR1 protein, either at the usual 152-kDa position or at any other region of the gel. The immunoprecipitates of ethanol-grown cells, in contrast, always produced a clearly detectable ADR1 signal (Fig. 5, lane 3) which was peptide blockable (Fig. 5, lane 4). Similar results were obtained with a strain carrying eight copies of the ADR1-5p gene and a strain expressing the ADR1-7p allele (S230L) (Fig. 5, lanes 5 to 8 and 9 to 12). Our results indicate that the enhanced ADR1 activity conferred by the ADR1p mutations is not attributable to an increase in ADR1 protein synthesis.

The conclusion that ADR1p mutations cause increased ADR1 activity by a mechanism which does not rely upon increased ADR1 protein translation is supported further by the results of our experiments with strain 411-12. Strain 411-12 contains 16 integrated ADR1 gene copies and produces ca. 80 to 90 mU of ADH II activity per ml when grown on glucose (12). Although cells from strain 411-12 produce only one-fourth of the amount of ADH II activity produced by the ADR1-5p single-gene-copy-containing strain (above), immunoprecipitates of glucose-grown 411-12 cells routinely produce a clearly visible ADR1 signal (Fig. 3, lane 16). These results indicate that the ADR1p mutations cause enhanced ADR1 activity by a mechanism independent of ADR1 protein synthesis.

Differences in ADR1 mRNA abundance contribute slightly to the regulation of the rate of ADR1 protein synthesis. We next sought to identify the step, or steps, leading to the production of the ADR1 protein which are responsible for the observed increase in the rate of ADR1 protein synthesis. To distinguish between ADR1 mRNA availability and other translational control mechanisms, we compared the levels of ADR1 mRNA in glucose- and ethanol-grown cells. Previous investigations of strain 411-40 (single ADR1 gene copy) and strains isogenic to 411-40 carrying multiple copies of the ADR1 gene indicated the level of ADR1 mRNA in glucose-grown cells to be comparable to or slightly less than that found in ethanol-grown cells (3, 12). Although these strains are identical or comparable to the ones used in the present investigation, to ensure consistency between all phases of our analysis we measured ADR1 mRNA levels by using the strains and growth conditions employed throughout our current investigation of the ADR1 protein abundance effect.

Figure 6 illustrates the results obtained with a Northern blot analysis of ADR1 mRNA for strains 411-40 (1 ADR1 gene copy) and 411-12 (16 ADR1 gene copies) grown on glucose (lanes G) and on ethanol (lanes E). As shown, for each of the strains, the levels of ADR1 mRNA were found to be only slightly elevated in the ethanol-grown cells. Densitometric analysis indicates that ADR1 mRNA, when normalized to rRNA, is twice as abundant under ethanol growth conditions as under glucose growth conditions. These results are essentially the same as those previously obtained by us (3, 12, 17) and others (4). It, therefore, appears that control mechanisms other than those controlling ADR1 mRNA abundance must be responsible for the greater part of regulating the rate of ADR1 translation.

The rate of ADR1 protein degradation does not contribute to differences in ADR1 protein synthesis. The greater part of the increase in ADR1 protein synthesis rate under ethanol growth conditions must result from either an elevated rate of ADR1 translation or a decreased rate of ADR1 protein degradation, or possibly both. To distinguish between these possibilities we examined rates of ADR1 protein degradation in glucose grown cells by pulse-labelling the cells in vivo with 35S-amino acids for 2 h and chasing the cells with nonradioactive methionine. Cells were removed at selected time points during the chase and analyzed for their content of labelled ADR1 and total yeast proteins. Samplings of cells taken just before and at times following the addition of
unlabelled methionine showed identical levels of 35S label incorporated into proteins, indicating that the chase with nonradioactive methionine efficiently blocked all further incorporation of 35S-amino acids into total yeast proteins (Fig. 7b). Minimal ADR1 protein degradation was observed during the first 2 to 3 h of the chase under glucose growth conditions (Fig. 7a). (The apparent decreased abundance of ADR1 at time zero in Fig. 7a was due to the antigenic ADR1 peptide being added during the immunoprecipitation; other experiments showed that ADR1 abundance did not significantly change between 0 and 1 h after the chase.) The data presented in Fig. 7 indicate a half-life of 3 to 4 h for ADR1 under glucose growth conditions. Significant differences in the rates of ADR1 protein synthesis were observed, however, in cell extracts pulse-labelled for only 15 min. An accelerated rate of ADR1 protein degradation, therefore, cannot account for the relatively low level of ADR1 protein present under glucose growth conditions. Indeed, the half-life of the ADR1 protein was actually found to be slightly shorter (about 2- to 3-h half-life) (Fig. 8) under ethanol growth conditions than under glucose growth conditions. This result confirms that differences in ADR1 degradation cannot significantly contribute to the differences in ADR1 protein abundance and indicates instead that differences in the rates of ADR1 protein translation must account for the higher rate of ADR1 protein accumulation observed for ethanol-grown cells.

Rate of ADR1 protein synthesis increases within 40 to 60 min after shifting cells to ethanol-containing medium. ADR1-dependent ADH2 transcription is known to be initiated within 1 h of depletion of glucose from the medium (14). We, therefore, examined the rate of ADR1 protein synthesis during the time period immediately following removal of glucose from the medium to determine whether the increased ADR1 protein translation rate is correlated with the time at which ADR1 activation is presumed to occur. Figure 9 illustrates that the rate of ADR1 protein synthesis increased dramatically in strain 411-3 (9 ADR1 gene copies)
within 40 to 60 min after shifting cells from glucose to ethanol growth conditions. At time points beyond the first 40 to 60 min (i.e., 220 min, 340 min, and 24 h) the rate of ADRI synthesis remained relatively constant (Fig. 9). Similar results were obtained for a strain (6-2) containing eight integrated copies of the ADRI-5' gene (data not shown). These results provide strong evidence that the glucose regulation of ADH2 expression by the control of ADRI protein synthesis is a physiologically significant control mechanism since ADH2 expression is known to be directly responsive to the dosage of ADRI (12).

The 510-nt 5' untranslated region of ADRI mRNA is not involved in ADRI translational control. We investigated the potential role of the 510-nucleotide (nt) 5' untranslated region of ADRI mRNA in the control of ADRI translation. While this region in ADRI lacks short open reading frames found to be important in controlling translation of the yeast GCN4 mRNA (26), we considered the possibility that other features contained within the region might mediate carbon source-dependent translation. To investigate this possibility, we replaced the 510-nt untranslated region of ADRI, including the upstream promoter sequences, with a truncated promoter derived from the glyceraldehyde 3-phosphate dehydrogenase (GAP) gene. The truncated GAP promoter was used because it is a low-expression promoter that would be expected to produce ADRI mRNA at levels equivalent to or only slightly higher than those normally expressed by the cell and because its efficiency is not regulated by the carbon source (27a).

Northern analysis indicated the level of G-ADRI mRNA in a yeast strain containing a single integrated dose of G-ADRI to be approximately 15-fold higher than that in the isogenic strain carrying a single ADRI gene copy (Fig. 6, first two lanes compared with the third and fourth lanes). Thus, G-ADRI produced ADRI mRNA in amounts which would be expected for a strain carrying about 15 copies of ADRI (12). We determined ADH II enzyme activity in the G-ADRI-carrying strain for glucose and ethanol growth conditions to be 50 and 5,000 mU/mg, respectively. These values are consistent with those expected for a strain carrying 10 to 12 copies of ADRI (12) and are in good agreement with the elevated levels of G-ADRI mRNA production illustrated in Fig. 6. The amount of G-ADRI mRNA in ethanol-grown cells was determined to be twice that found under glucose growth conditions. In contrast, we determined the amount of ADRI protein immunoprecipitated from 35S-amino acid extracts from the G-ADRI-carrying strain to be 10- to 15-fold greater under ethanol growth conditions than under glucose growth conditions (Fig. 3, compare lanes 12 and 13), consistent with our above analysis of strains containing one or more copies of the complete ADRI gene. These surprising results indicate that the ADRI translation rate differences under the glucose and ethanol growth conditions are retained when the 510-nt 5' untranslated region of ADRI mRNA is replaced with a different sequence and that this region is, therefore, not involved in the glucose regulation of ADRI translation.

ADRI coding sequences corresponding to amino acid residues 262 through 642 are involved in ADRI translational control. Having determined that the 5' untranslated region of ADRI mRNA does not control the ADRI translation rate,
The ADR1-642 protein of the ADR1 species was radiolabelled during glucose growth conditions (data not shown), indicating that the majority of the 262 amino acids were intact. Although the observed abundance differences of the individual forms of ADR1-262 between glucose and ethanol growth conditions were not reproducible, the total amount of ADR1 protein (both forms together) always remained equivalent under the two growth conditions. The ADR1-262 mRNA levels were found to be similar under ethanol and glucose growth conditions (data not shown), results which are consistent with the ADR1 protein synthetic rates determined for ADR1-262 and the ADR1 mRNA patterns observed for all of the strains investigated in this study. The translational control of ADR1 appears mediated, therefore, by sequences localized within the coding region of the transcript corresponding to amino acid residues 262 through 642 of the ADR1 protein.

**DISCUSSION**

The results of our investigation demonstrate that the rate of protein synthesis for the transcriptional activator ADR1 is 10- to 16-fold greater under ethanol growth conditions than under glucose growth conditions. This derepression in ADR1 protein translation was found to occur within 40 to 60 min of depleting cells of glucose, the same time frame defined previously for the commencement of ADR1-dependent ADH2 transcription (14). Our results indicate that glucose represses ADH2 expression by reducing the rate of ADR1 mRNA synthesis under repressed conditions. This previous observation that linear increases in ADR1 dosage result in corresponding linear increases in the amount of ADR1 mRNA and in ADH2 expression under glucose growth conditions (12). Strains containing ADR1 alleles display reduced levels of ADR1 protein synthesis under repressed conditions in a manner similar to that observed for strains expressing the wild-type ADR1 allele. We, therefore, conclude that the mechanism by which ADR1 alleles cause increased ADH2 transcription must be distinct from that responsible for effecting changes in the rate of ADR1 protein translation.

Our previous studies indicate that yeast strains expressing a single copy of the ADR1 allele show an eightfold increase in ADH2 transcription within 1 h of shifting cells from glucose to ethanol growth conditions (14). Our current data suggest that this increase is a direct result of increased ADR1 protein synthesis signalled by glucose depletion. Interestingly, strains carrying eight copies of an ADR1 allele display glucose-repressed ADH II levels that are nearly equivalent to the level of ADH II found under ethanol growth conditions in an isogenic strain containing a single wild-type ADR1 allele (12). We interpret these results to suggest that the overall derepression of ADH2 results from the combined effects of increased ADR1 protein translation rates and some other activation of the already synthesized ADR1 protein (equivalent to an ADR1 allele). The posttranslational activation mechanism may, as previously suggested, be triggered by a dephosphorylation event (7, 16). It remains possible, however, that as yet undefined mechanisms contribute to the regulation of ADH2 transcription in addition to control of ADR1 protein translation.

The half-life of ADR1 protein was found to be roughly equivalent under glucose and ethanol growth conditions, indicating that the observed regulation of ADR1 is independent of ADR1 degradation rates. This point is further sup-

![Graph](http://mcb.asm.org/)

**FIG. 10.** Identification of the regions controlling ADR1 translation. Cells were grown and radiolabelled as described in the legend to Fig. 4 and Materials and Methods. Lanes 9 and 10 were radiolabelled during glucose growth conditions with $^{[35]S}$S-amino acid labelling and analysed for new ADR1 protein synthesis. Strain B19 carries 21 copies of ADR1-642, while strain 35 carries 21 copies of ADR1-262 (2). (The numbers 642 and 262 correspond to the numbers of N-terminal ADR1 amino acids in the truncated ADR1 protein products expressed in each strain.) The ADR1-642 protein from strain B19 gave a boldly visible signal following growth on ethanol-containing medium but was not detected in glucose-grown cells (Fig. 10, lanes 3 and 2, respectively). These results are similar to those observed for the full-length ADR1 protein and would be expected if the translational control mechanism does not become disrupted as a result of removal of over half of the ADR1 coding sequence (681 C-terminal residues). Similar results were obtained with a yeast strain containing only nine copies of ADR1-642 (data not shown). Also, the amounts of ADR1-642 mRNA, when normalized to rRNA levels, were found to be only slightly elevated in ethanol-grown cells compared with glucose-grown cells, data consistent with our observations for strains containing the full-length ADR1 gene.

The ADR1-262 protein, in contrast to ADR1-642 and ADR1-1323, was observed to yield boldly visible signals under both glucose and ethanol growth conditions. Densitometric analysis indicated ADR1-262 protein, though present in two forms, to be equally abundant in glucose- and ethanol-grown cells (Fig. 10, lanes 3 and 7). We presume the two forms of ADR1-262, which migrate in the 40-kDa region of the SDS-polyacrylamide gel, to be due to the occurrence of multiple phosphorylated species (both species are phosphoproteins; Fig. 10, lanes 9 and 10) or possibly to proteolysis. Antibodies directed against ADR1 peptides corresponding to regions 208 to 231 and 2 to 21 immunoprecipitated both forms with equal efficiencies (data not shown), indicating that the majority of the 262 amino acids were intact. Although the observed abundance differences of the individual forms of ADR1-262 between glucose and ethanol growth conditions were not reproducible, the total amount of ADR1 protein (both forms together) always remained equivalent under the two growth conditions. The ADR1-262 mRNA levels were found to be similar under ethanol and glucose growth conditions (data not shown), results which are consistent with the ADR1 protein synthetic rates determined for ADR1-262 and the ADR1 mRNA patterns observed for all of the strains investigated in this study. The translational control of ADR1 appears mediated, therefore, by sequences localized within the coding region of the transcript corresponding to amino acid residues 262 through 642 of the ADR1 protein.
ported by our observation that the ADR1 half-life (3-4 h on glucose) is much longer than the time required to visualize ADR1 protein differences in the amounts of newly synthesized protein during a pulse-labelling experiment (15 min). We have also demonstrated that the long untranslated 5' leader sequence of ADR1 mRNA is not involved in controlling ADR1 protein synthesis. Instead, coding sequences corresponding to amino acid residues 262 through 642 were found to be required for regulating ADR1 protein synthesis. The potential role of the 5' untranslated leader sequence of ADR1 mRNA in controlling other processes, such as mRNA stability, remains to be investigated.

Our previous deletion analysis of the ADR1 gene (2) did not reveal internal regions involved in glucose regulation of ADR1 protein synthesis due to the diminished capacity of the truncated ADR1 forms to activate ADH2. ADR1-262 protein, for example, would be expected to produce 10- to 16-fold more ADH II activity than ADR1-642 under glucose growth conditions on the basis of their respective rates of protein synthesis. However, one copy of ADR1-642 is about four- to sixfold more active than ADR1-262. An increase in ADR1-262 protein abundance relative to ADR1-642 due to increased translation would, therefore, have been masked by its diminished intrinsic activity with respect to ADH2 activation.

The molecular mechanism by which glucose reduces the rate of ADR1 translation, other than the small effect of ADR1 mRNA levels, remains unclear. None of the genes known to affect ADH2 expression, including CCR1, ADR6, CCR4, CRE1, and CRE2 (9, 11, 30) appear to be involved in controlling ADR1 mRNA translation. Previous searches for trans-acting genes that affect ADH2 expression under glucose growth conditions have identified only ADR1c mutations or mutations in the CRE genes, the latter of which act independently of ADR1 in controlling ADH2 expression (11). Interestingly, the sequence in ADR1 between nt 840 and 869 (corresponding to amino acids 281 and 290) predicts a perfect 13-nt stem and 4-nt loop structure. It remains possible that such a stem-loop might operate to impede translation directly or to serve as a binding site for a protein that, in turn, functions to regulate ADR1 translation rate. Deletion of this region, however, appears to have no effect on ADR1-dependent ADH2 expression (unpublished observations). Other sequences that could potentially control ADR1 translation have not been identified.

Our results indicate that the overall repression of ADH2 by glucose results from a combination of factors, including effects on ADR1 RNA abundance, protein translation, and posttranslational activity. The existence of a single on-off switch controlling glucose repression in yeast, therefore, is unlikely. Instead, contributions from several mechanisms accrue to produce the 500-fold difference in ADH II enzyme levels observed between glucose and ethanol growth conditions. The occurrence of such a multicomponent system suggests an accretion of regulatory mechanisms during the evolution of ADH2 regulation. The various components may represent historical additions that are mechanistically and perhaps evolutionarily unrelated to each other. The only comparably studied system with respect to the ADR1/ADH2 system in yeast cells involving glucose repression is that of the galactose-metabolizing genes. In this system, at least several different control mechanisms have been identified to be responsible for the differences amounting to the 1,000-fold change in GAL gene expression observed under contrasting growth conditions (20).

A recent report (31) has suggested that ADR1 protein levels are equivalent under glucose and ethanol growth conditions. This study for the most part, however, utilized strains containing plasmid-borne copies of the ADR1 gene. The possibility therefore exists that glucose-induced increases in ADR1 plasmid dosage (in order to maintain the high ADH II activity required for fermentative growth) (12) and ethanol-induced reductions in ADR1 plasmid dosages (in order to reduce ADR1-induced petite formation) (6) might have influenced the results. Because in this other study (31) neither the relative levels of ADR1 mRNA nor the rates of ADR1 protein degradation or synthesis were analyzed for the one strain not containing plasmid-borne copies of ADR1, it is not possible to fully interpret the significance of these results with respect to ours. The regulation of ADR1 protein synthesis could be strain dependent since it has been observed that a couple of yeast strains, in contrast to the vast majority of strains analyzed (3, 4, 12, 17) (Fig. 6), display much reduced ADR1 mRNA levels under glucose growth conditions (4). We have analyzed one of these strains (4) and have found that the greatly reduced ADR1 mRNA levels are the result of at least several different factors not normally present in the yeast strains we commonly use (unpublished observations). To ensure consistency in all of our results and to avoid possible strain-dependent differences, our analysis of glucose regulation of ADH2 (2, 7, 12, 17) has utilized isogenic or very closely related strains.

Our results indicate that ADR1 turns over with a half-life of about 3 to 4 h under glucose growth conditions. Other yeast proteins as observed in Fig. 6a and b, appear to be significantly more stable than ADR1. ADH I and ADH II also have relatively long half-lives in comparison to ADR1, on the order of at least 20 hours (10a, 19). We have also observed that the transcriptional activator CCR4 displays a half-life of 7 to 9 h (unpublished observations). The relatively short half-life of ADR1 protein suggests a mechanism for controlling cellular levels of ADR1 and for removing altered or damaged forms of ADR1 which may potentially decrease the efficiency of ADH2 regulation. It is unclear whether the relatively rapid degradation observed for ADR1 is generally true for other transcriptional factors or whether such a mechanism contributes to the low cellular levels observed for such proteins. Such a mechanism might minimize the deleterious effects known to result from their overproduction in yeast cells (6, 21).

We also have demonstrated that ADR1 is a phosphoprotein. Phosphorylation of ADR1 occurred at multiple sites to generate multiple forms of the ADR1 protein that were distinguishable on the basis of their SDS-PAGE migration patterns and in their sensitivity to in vitro dephosphorylation by alkaline phosphatase. Phosphorylation of ADR1 was found to occur in cells pulse-labelled for only 15 min (data not shown), indicating that it occurred either during ADR1 translation or soon after translation was completed. The pattern of ADR1 phosphorylation also appeared to be the same in ethanol- and glucose-grown cells on the basis of SDS-PAGE analysis.

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