Isolation and Characterization of Further *Cis*- and *Trans*-Acting Regulatory Elements Involved in the Synthesis of Glucose-Repressible Alcohol Dehydrogenase (ADHII) in *Saccharomyces cerevisiae*

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Summary. Starting with yeast cells lacking the constitutive alcohol dehydrogenase activity (ADHI), mutants with partially glucose-insensitive formation of ADHII were isolated. Genetic analysis showed that four mutants (designated $ADR3^c$) were linked to the ADHII-structural gene, $ADR2$, and were *cis*-dominant. On derepression, two of them produced elevated ADHII-levels, indicating a promotor function of the altered controlling site. The other $ADR3^c$-mutant alleles affected the ADHII-subunit association in diploids carrying two electrophoretically distinct alleles of the structural gene $ADR2$. Twelve semidominant constitutive mutants could be attributed to gene $ADR1$ ($ADR1^c$-alleles) previously identified by recessive mutants with blocked derepression. This suggested a positive regulatory role of the $ADR1$ gene product on the expression of the ADHII-structural gene. A pleiotropic mutation $ccr1$ (Ciriacy, 1977) was epistatic over glucose-resistant ADHII-formation caused by $ADR1^c$-alleles. From this it was concluded that $CCR1$ specifies for a product co-activating the structural gene or modifying the $ADR1$-gene product. A further regulatory element (gene designation $ADR4$) not linked to the structural gene could be identified upon isolation of recessive constitutive mutants $adr4$ from a $ccr1$ $ADR1^c$-double mutant.

Introduction

Investigations of the genetics of different molecular forms of alcohol dehydrogenase in the yeast *Saccharomyces cerevisiae* (Ciriacy, 1975a, 1975b; Wills and Phelps, 1975) have revealed at least four unlinked genes involved in the formation of three ADH isozymes. Based on genetic and biochemical evidences, genes $ADC1$ and $ADR2$ were thought to be structural genes coding for ADHI- and ADHII-polypeptides

(see Ciriacy, 1976). Recently the existence of two different structural genes for the constitutive isozyme (ADHI) and the glucose-repressible isozyme (ADHII) has been definitely demonstrated by Wills and Jörnvall (1979) by sequencing of the respective polypeptides. ADHII has gained a specific attraction as its formation is under control of carbon catabolite repression which is considered as a central regulatory system of controlling the synthesis of many enzymes of carbon metabolism (see e.g. Ciriacy, 1978, and references therein). Starting with strains lacking the constitutive and mitochondrial ADH, mutants with glucose-resistant ADHII-formation could be selected (Ciriacy, 1976). Modification of the original selection procedure led to the identification of further dominant and recessive regulatory mutant alleles of genes involved in the expression of the ADHII-structural gene. Four new *cis*-dominant ADHII-constitutive mutant alleles of gene $ADR3$ were found. They differed from the previously described $ADR3^c$-mutants (Ciriacy, 1976). Based on the results reported in this paper, a preliminary model of regulation of the ADHII-structural gene expression in yeast is discussed.

Materials and Methods

with less than 5×10^6 cells/ml medium and harvested before glucose was exhausted from the medium. All cultures were grown on a rotatory shaker at 28° C Antimycin A (dissolved in ethanol, final concentration 1 ppm) and allylalcohol (final concentration 10 mM) were added to YEPD-media after autoclaving.

Preparation of Crude Extracts, Enzyme Electrophoresis and Enzyme Assay. Cells were broken with glass beads. Electrophoretic separation of crude extracts, visualization of ADH activity on starch gels, and spectrophotometric determination of ADH activity were carried out as described previously (Ciriacy, 1975a). Protein was determined according to Lowry et al. (1951) with bovine serum albumin as standard.

Mutagenesis and Genetic Analysis. All mutants described under Results were obtained by treatment of haploid cells with 1% ethylmethane sulphonate in 0.1 M phosphate buffer, pH 7.0 at 28° C or by irradiation with an UV-lamp causing about 40–60% killing. Methods for mating, sporulation and tetrad analysis have been described previously (Ciriacy, 1975b).

Results

Isolation of Mutants. The screening method for yeast mutants with constitutive synthesis of ADHII described previously (Ciriacy, 1976) was improved by adding 1 ppm antimycin A to a solid YEPD 8% medium. This antibiotic blocks respiration of yeast cells (Chance, 1972) and completely prevents all residual growth of ADHI⁻cells (Ciriacy and Breitenbach, 1979). Haploid cells of the genotype *adc1-11 adm ADR1 ADR2-F* (no ADHI and m-ADH, ADHIIF repressible by glucose) were mutagenized and spread onto YEPD 8%+1 ppm antimycin A plates. After five days a few colonies had appeared from which cells were isolated and grown on liquid YEPD 8% medium for crude extract preparation. Altogether, 350 clones were then tested for ADH activity by enzyme electrophoresis. Clones with an ADH band differing in mobility from the ADHIIF of the parent strain (Fig. 1, lane 1) were discarded as well as those showing no visible band on the gel. Finally, fourteen

mutants were selected for a further genetical and biochemical analysis. Two additional mutants (R414 and R416) were derived by the same method from a strain of the genotype *adc1-11 adm adr1-1 ADR2-F.*

Trans-Acting Genes. Twelve of the sixteen antimycin-resistant mutants were allelic to gene *ADR1* previously identified by recessive mutations with reduced derepression of ADHII (Ciriacy, 1975b). Enzyme determinations showed (Table 1) that *ADR1ᶜ*-mutants on glucose had only 10–20% of the derepressed wild-type activity, whereas derepressed enzyme levels in mutant cells were 2–3 times higher than in the wild type. All *ADR1ᶜ/ADR1* diploids were antimycin-resis-
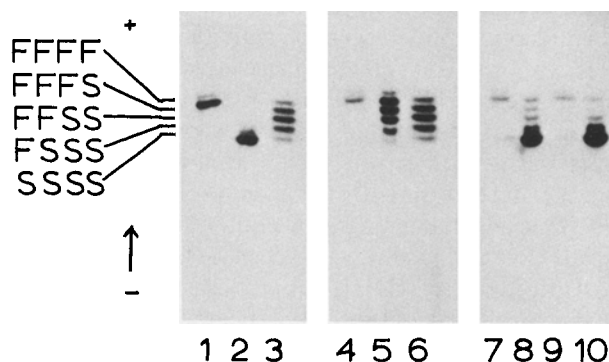

Fig. 1. Enzyme electrophoresis of ADHII on starch gels, original scale. Designations FFFF, FFFS, etc. refer to subunit composition of the respective bands. In front of the main bands usually a weak sub-band appears (cf. lanes *1, 2, 5*), which is considered as a derivative of the respective ADHII-band. Crude extracts were prepared from cells grown either in YEPD 8% indicated below as *r* (repressed cells) or in YEP-3% ethanol, indicated as dr (derepressed cells). About 20 µg protein was applied to the gel. Lane *1*: haploid wild-type, *ADR2-F*, dr; *2*: *ADR2-S*, dr; lanes *3* and *6*: diploid wild-type, *ADR2-F/ADR2-S*, dr; *4*: *ADR3-4ᶜ ADR2-F/ ADR3 ADR2-S*, r; *5*: same as 4, but dr; *7*: *ADR3-6ᶜ ADR2-F/ ADR3 ADR2-S*, r; *8* same as 7, but dr; *9*: *ADR3-7ᶜ ADR2-F/ ADR2-S*, r; *10* same as 9, but dr

Table 1. Specific activity of ADHII and growth characters of wild type and various mutants altered in *trans*-acting regulatory elements. Repressed cells (r) were grown on YEPD 8%; derepressed cells (dr) were obtained from YEP-3% ethanol cultures. Activities were calculated as nmoles × min⁻¹ × mg protein⁻¹

Strain	Genotype	Specific activity		Growth on YEPD	
		r	dr	+antimycin	+allylalcohol
43-2B	*ADR1*	15	1,850	−	+
CH1-9C	*adr1-1*	5	90	−	+
R234	*ADR1-5ᶜ*	414	5,400	+	−
diploid	*ADR1/ADR1-5ᶜ*	118	4,970	+	−
53.3-1C	*ADR1 ccr1*	5	25	−	+
segregants	*ADR1-5ᶜ ccr1*	30	1,040	−	+
R516	*ADR1-5ᶜ ccr1 adr4-516*	802	2,800	+	−
segregants	*ADR1-5ᶜ CCR1 adr4-516*	1,180	n.t.	+	−

tant and sensitive to allylalcohol on YEPD-medium, but enzyme activities in such heterozygous diploids were significantly lower than in haploid mutants (Table 1, line 4). On this basis, $ADRI^c$ alleles were considered as semi-dominant.

The availability of mutant alleles specifically regulating the level of ADHII allowed to test the influence of other regulatory elements involved in carbon catabolite repression. Of the various genes identified (Ciriacy, 1977, 1978), the recessive mutant allele $ccr1$ was selected for further analysis. $ccr1$ causes a loss of the ability to derepress enzymes of the glyoxylate shunt, ADHII and gluconeogenic enzymes, and to ferment maltose (Ciriacy, 1977). Remarkably, $ADRI^c$ $ccr1$ double mutants had lost the ability to form substantial amounts of ADHII on glucose. Accordingly these cells were sensitive to antimycin and resistant to allylalcohol on YEPD, i.e. $ccr1$ was epistatic over $ADRI^c$. On the other hand, on a derepression medium (YEPE) ADHII showed some increase in $ADRI^c$ $ccr1$ whereas this was completely prevented in $ADR1$ $ccr1$ cells (cf. Table 1, lines 5 and 6).

The inability of $ADRI^c$ $ccr1$ double mutants to grow on YEPD+antimycin allowed for the selection of mutants in which ADHII-synthesis no longer depended on the function of $CCR1$. Selection was carried out in the same manner as described for $ADRI^c$ mutants. In order better to recognize $ccr1$-revertants, antimycin-resistant colonies were replica plated on synthetic media with ethanol as sole carbon source providing a growth criterion for the $ccr1$ mutant allele. Finally twelve clones still unable to grow on synthetic ethanol medium but resistant on YEPD +antimycin were selected for a further analysis. ADHII was again partially derepressed on glucose. The data obtained with a representative isolate R516 are given in Table 1, line 7. ADHII was low in all mutant × $ccr1$ $ADRI^c$ diploids grown on glucose, indi-

cating recessiveness of the new mutant alleles which were allelic to each other. A genetic analysis revealed that the reversions had neither occurred at a site linked to the ADHII structural gene $ADR2$ nor to gene $ADR1$. Furthermore, the mutant allele (gene designation $adr4$) is very probably only expressed in combination with an $ADRI^c$-allele but not in combination with the $ADR1$ wild-type allele. ADHII was not significantly higher when $adr4$ was in combination with an intact $CCR1$-allele (Table 1, line 8).

Cis-dominant Mutants, $ADR3^c$. Two revertants (R22 and R112) from the $ADR1$ $ADR2$-F parent and both isolates from the $adr1$-1 $ADR2$-F strain (R414 and R416) were closely linked to the ADHII-structural gene, $ADR2$. This could be shown by crossing the revertants to a wild type carrying the S-allele of the structural gene ($ADR2$-S), and by tetrad analysis of the resulting diploids. In addition to that, on glucose medium mutant × wild-type diploids synthesized only ADHIIF (Fig. 1, lanes 4, 7 and 9), i.e. glucose-resistant synthesis was cis-dominant (designation of mutant alleles: $ADR3^c$). ADHII levels in glucose-repressed cells were similar for all newly isolated $ADR3$-mutants (Table 2, first column), but marked differences were observed with regard to ADHII-levels in derepressed cells. $ADR3$-4 and $ADR3$-5^c had higher activities than the wild-type, whereas in $ADR3$-7^c cells derepressed activities were not higher than in glucose-grown cells. A comparison of the electrophoretic patterns of various derepressed diploids heterozygous for the structural gene also revealed such differences. $ADR2$-$F/ADR2$-S wild-type diploids normally show an isozyme pattern with five bands and with a symmetrical distribution of activity, e.g. slow and fast band of the pattern had a similarly low activity (Ciriacy, 1975 b, and Fig. 1, lanes 3, 6). In the derepressed double heterozygote $ADR3$-4^c $ADR2$-$F/ADR3$ $ADR2$-S more activity was found in the F-band than in the

Table 2. Effect of various controlling site mutations ($ADR3^c$) on glucose-repressed (r) and derepressed (dr) levels of ADHII. Values in parentheses: Variation observed within 8–12 meiotic segregants

Strain	Relevant genotype	Specific activity	
		r	dr
43-2B	$ADR1$ $ADR3$	15	1.850
CH1-9C	$ad1$-1 $ADR3$	5	90
R22	$ADR1$ $ADR3$-4^c	463 (270–770)	4,440 (2,625–5,680)
R112	$ADR1$ $ADR3$-5^c	410 (304–434)	4,680 (4,180–6,920)
R414	$adr1$-1 $ADR3$-6^c	614 (367–847)	704
Segregants	$ADR1$ $ADR3$-6^c	783	1,590
R416	$adr1$-1 $ADR3$-7^c	497 (401–681)	428
Segregants	$ADR1$ $ADR3$-7^c	522 (354–683)	746 (556–893)

S-band, this very probably is a result of an enhanced expression of the *ADR2-F* allele as compared to its wild-type counterpart (Fig. 1, lane 5). In contrast to this, derepressed *ADR3-6^c/ADR3* and *ADR3-7^c/ADR3* diploids showed rather low activities of those bands which consisted of more than one F-subunit (FFFF, FFFS and FFSS, Fig. 1, lanes 8 and 10). While the observed distribution of activities may be consistent with the low derepressed expression in haploid *ADR3-7^c* cells such a pattern was unexpected in the case of the *ADR3-6^c* allele which showed nearly wild-type activity in derepressed haploid cells. To put it in a formal terms, the *ADR3* wild-type allele suppressed to some extent the expression of the mutant allele *ADR3-6^c*. Furthermore, relative activities of the various hybrid and parental bands (Fig. 1, lanes 8 and 10) revealed the subunit association did not follow a random distribution, e.g. the FFFF-bands had a similar activity as the FFFS-band, whereas the expected ratio would be less than $1:12$ assuming F- and S-subunits were present in a ratio of less than $1:3$. It was concluded from these results that all *ADR3^c* alleles expect *ADR3-4^c* and *ADR3-5^c* did not only cause glucose-resistant formation of ADHII but influence also the association of slow and fast subunits to hybrid forms or the activity of these hybrid enzymes.

Discussion

The results presented in this paper demonstrate that the expression of the ADHII structural gene *ADR2* in yeast is under the control of a least four unlinked genetic elements: *ADR3, ADR1, CCR1* and *ADR4*. They were characterized by genetic and biochemical analyses of mutants with partially constitutive ADHII-synthesis *(ADR3^c, ADR1^c* and *adr4)* or with a lack in derepression of ADHII *(adr1* and *ccr1*; see Ciriacy, 1975b; 1977).

An interpretation of the various mutant classes with regard to their gene functions is comparatively straightforward in the case of *ADR3^c*-mutant alleles. *Cis*-dominance and tight linkage to the ADHII-structural gene demonstrate that *ADR3* is the controlling site of gene *ADR2*. The various *ADR3^c*-mutant alleles however differ remarkably with respect to the derepressed activity of the structural gene *ADR2*. They could roughly be divided in two subclasses: Two of them *(ADR3-4^c* and *ADR3-5^c)* caused higher than normal derepressed ADHII-levels, whereas all others had wild-type or even much lower levels (Ciriacy, 1976, and this article). The latter subclass could further be characterized by a reduced expression of the mutated sequence *ADR3^c-ADR2* in derepressed mu-

tant × wild-type diploids. Moreover, the mutational alterations seem to interfer with the association of the slow and fast subunits in *ADR2-S/ADR2-F* diploids. This suggests that the *ADR3* region consists of at least two functionally different sites: one can be thought of as a promotor site, represented by mutant alleles *ADR3-4^c* and *ADR3-5^c* which showed a "promotor-up" effect in derepressed cells. A second *ADR3*-region is represented by the other five *ADR3^c*-mutant alleles. The function of this site is still unknown; there are however, some suggestions that this site interacts with the structure of the polypeptide itself.

Another group of partially constitutive mutants has been isolated from wild-type cells. They were allelic to a mutant allele previously described *(adr1-1;* Ciriacy, 1975b) causing strongly reduced derepression of ADHII. *ADR1^c* alleles led to an overproduction of ADHII in the derepressed condition. Glucose-repressible enzymes other than ADHII were not affected in *adr1* or *ADR1^c*-mutants. These data are consistent with the assumption of *ADR1* being a positive regulatory gene specifically activating the expression of the structural gene *ADR2*. This expression was reduced in glucose-repressed *ADR1/ADR1^c* heterozygotes but still higher than in pure wild-type cells. This indicates that the wild-type *ADR1* gene product is even present in glucose-repressed cells. Assuming that *ADR1* codes for a multimeric protein, incomplete dominance of *ADR1^c*-mutations can be considered as a result of interactions between mutant and wild-type gene products (cf. Zimmermann and Eaton, 1974). To explain the regulatory role of the *ADR1* gene product, it is suggested that in glucose-repressed wild-type cells the *ADR1*-gene product is modified to an inactive form. This inactivation of the regulatory protein is no longer possible in *ADR1^c*-mutants resulting in a slight but permanent activation of the structural gene even under glucose repression.

There are at least two further trans-acting regulatory elements involved in the expression of *ADR2*. Gene *CCR1* was previously identified by a recessive mutant allele *ccr1* unable to derepress various glucose-sensitive functions including ADHII (Ciriacy, 1977). *ccr1 ADR1^c* double mutants had again low repressed levels of ADHII. This clearly shows that the activating effect of the *ADR1* gene product depends on the function of gene *CCR1*. This gene codes for a constitutive protein which either activates the structural gene *ADR2*, or, alternatively activates the *ADR1* regulatory protein, which in turn than activates *ADR2*. A fourth regulatory gene could be identified upon isolation of constitutive mutants *adr4* (wild-type designation *ADR4*) from the glucose-repressible *ccr1 ADR1^c* double mutant. Because of the complete reces-

siveness, *ADR4* specifies a negative regulatory factor (see Beckwith and Rossow, 1974). Under glucose-repression, *adr4*-mutant gene product(s) can replace the function of gene *CCR1*, but constitutive formation of ADHII seems to depend on the presence of a constitutive *ADR1ᶜ* regulatory allele. It can not yet clearly be established whether *ADR4* acts specifically on the *ADR1* or *ADR2* gene, or whether its gene product constitutes a regulatory element with a pleiotropic action spectrum.

The main purpose of our work concerning ADHII-formation was to design a model of a genetic regulatory system in yeast. Some features of the model discussed here are still speculative; for instance, it is assumed that gene regulation takes place at the level of transcription. As far as I am aware, this has been proved in yeast for glucose-sensitive enzyme synthesis only in one case (cytochrome c: Zitomer and Nichols, 1978). The results presented here convey however, some ideas of the multiple *regulatory* elements acting on the formation of a single protein in yeast.

References

Chance, B.: The nature of electron transfer and energy coupling reactions. FEBS Lett. **23**, 3–12 (1972)

Ciriacy, M.: Genetics of alcohol dehydrogenase in *Saccharomyces cerevisiae*. I Isolation and genetic analysis of *adh* mutants. Mutat. Res **29**, 315–326 (1975a)

Ciriacy, M.: Genetics of alcohol dehydrogenase in *Saccharomyces cerevisiae*. II. Two loci controlling synthesis of the glucose-repressible ADHII. Mol Gen. Genet. **138**, 157–164 (1975b)

Ciriacy, M.. *Cis*-dominant regulatory mutations affecting the formation of glucose-repressible alcohol dehydrogenase (ADHII) in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **145**, 327–333 (1976)

Ciriacy, M.: Isolation and characterization of yeast mutants defective in intermediary carbon metabolism and in carbon catabolite derepression. Mol Gen. Genet. **154**, 213–220 (1977)

Ciriacy, M.: A yeast mutant with glucose-resistant formation of mitochondrial enzymes Mol. Gen. Genet. **159**, 329–335 (1978)

Ciriacy, M , Breitenbach, I.: Physiological effects of seven different blocks in glycolysis of *Saccharomyces cerevisiae*. J Bacteriol. **139**, 152–160 (1979)

Lowry, O.H., Rosebrough, H.J., Farr, A.L., Randall. R.J.: Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**, 265–275 (1951)

Wills, C., Jörnvall, H.: The two major isozymes of yeast alcohol dehydrogenase. Eur. J. Biochem. (1979, in press)

Wills, C., Phelps, J : A technique for the isolation of yeast alcohol dehydrogenase mutants with altered substrate specificity. Arch. Biochem Biophys. **167**, 627–637 (1975)

Zimmermann, F.K., Eaton, N.R.. Genetics of induction and catabolite repression of maltase synthesis in *Saccharomyces cerevisiae*. Mol Gen. Genet. **134**, 261–272 (1974)

Zitomer, R S., Nichols, D L.· Kinetics of glucose repression of yeast cytochrome c. J. Bacteriol. **135**, 39–44 (1978)